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EXPERIMENTAL GENERALIZED ANALGESIA AFTER EXPOSURE TO SOME WAR GASES.*

By JOHN AUER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 4 TO 6.

(Received for publication, June 28, 1921.)

During a series of investigations, taken up at the instance of the Surgeon General of the Army, concerning the functional effects following exposure to dimethylsulfate and chloropicrin in a gaseous or finely subdivided state, it was noted accidentally that these two war gases reduced pain perception to an astonishingly low level. This analgesia was so profound in many cases that a laparotomy could be performed without any indication of pain and without markedly influencing the blood pressure. As this effect seems to be practically unknown, numerous experiments were carried out to test pain perception in gassed cats. The results justify the statement that the two war gases mentioned strikingly reduce pain perception in cats under the conditions which will be described.

The only reference in the literature of war gases bearing on this aspect which I have been able to find, is an article by Naiding.[1] This author had the opportunity of examining soldiers gassed with chlorine and phosgene, and states, among other observations, that numbers of them exhibited a general or localized loss of pain perception; thus,

* A preliminary note was published in the *Proceedings of the Society for Experimental Biology and Medicine* (Auer, J., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 104). The final publication was delayed by conditions beyond the control of the author.

This work forms part of some war gas investigations which began in 1917 and were undertaken at the suggestion of Dr. Simon Flexner and Dr. Henry D. Dakin. I am under special obligation to Dr. Dakin for numerous helpful suggestions concerning methods of gassing, precautions to be observed, and also for kindly supplying me with chloropicrin.

[1] Naiding, M. N., *Russk. Vrach*, 1917, xvi, 391.

he noted that the analgesia involved the skin of the whole body in some, while in others it was localized in the forehead, scalp, hands, or feet. There is no record that deep pain perception was tested.

Methods.

Cats only were utilized in this investigation for reasons of convenience. The gas chamber was of the ice box type with a cubic capacity of 200 liters. In two of the walls large glass windows were sealed so that the entire chamber could be readily inspected at any time; fogging of the glass panes was prevented by using a commercial soap sold for this purpose. One side of the chamber was formed by a completely removable door. Gas tightness was obtained by carefully filling all visible cracks in the wall of the inner chamber with putty and then applying several coats of white enamel. The door was made gas-tight by providing two soft rubber gaskets, one glued on the door and the other to the box. Before closure the contact surfaces were covered with vaseline. Complete contact was assured by four lever handles on the box which fitted in corresponding hooks on the door.

The cats were gassed in a wire cage, this container being placed on brackets in the chamber. The brackets were about 5 inches from the floor of the chamber so that the animal was at the same level as the window during the gassing period. Under the wire cage an enameled pan was placed to prevent soiling of the chamber.

The heat necessary for vaporizing the dimethylsulfate or chloropicrin was furnished by an electric hot-plate which could be set to reach 250°C. This plate was located in one corner on the floor and its switch was placed on the outside of the chamber. The hot-plate also served as a convenient means of maintaining the chamber temperature between 25° and 30°C. The temperature was read from a thermometer suspended near a window in the chamber.

As the gases employed were heavier than air, a small motor-driven fan was screwed in a corner of the chamber. This fan was kept in motion constantly throughout the experiment and prevented the gas vapors from settling to the floor.

The duration of gassing was kept constant for each of the various series of experiments. With dimethylsulfate the cats were gassed for 1 hour in the great majority of experiments. With chloropicrin the duration of gassing was either ½ or 1 hour in most cases.

The concentration of gas chosen was such that death resulted in a majority of the animals within 1 to 4 days after gassing. This was determined by a series of trials and was as follows for the two gases: for dimethylsulfate 4.5 to 10,000 air; for chloropicrin 1 to 10,000 air.

The amount of liquid necessary to produce the required concentrations was readily calculated. Thus 20 cc. of gas were needed to give 1 to 10,000 concentration in a chamber of 200 liters capacity. As the molecular weight in grams of each liquid produces roughly 22 liters of gas, the weight of liquid required for 20 cc. of gas was determined and then translated into measure by volume. Curiously enough, the volumes of dimethylsulfate and chloropicrin necessary to produce

20 cc. of gas were identical: 0.087 cc. for dimethylsulfate and 0.088 cc. for chloropicrin. For practical purposes 0.09 cc. was considered the amount necessary for a 1 to 10,000 concentration. No corrections were made for temperature.

From these conditions it will be seen that the calculated concentration of the war gas would only be maintained for a short time, because condensation and droplet formation would begin shortly after vaporization. The gas concentration would then necessarily sink. This was not considered a drawback because the same conditions were maintained as far as possible throughout all the experiments; moreover, it is believed that this arrangement presents a closer approximation to conditions existing on the battle-field after the explosion of gas shells, than the ordinary procedure in which the desired gas concentration is drawn through the testing chamber.

Dimethylsulfate, $(CH_3)_2SO_4$, is a liquid at ordinary temperatures with a specific gravity of 1.32 and a boiling point of 188°C. Chloropicrin, or trichlornitromethane (CCl_3NO_2), is also a liquid and possesses a specific gravity of 1.69 and a boiling point of 112.8°C.

The chamber was evacuated by suction after the gassing period, the door of the chamber being perforated by two stop-cocks with $\frac{1}{4}$ inch lumen. These cocks were located in the upper and lower parts of the door respectively. The chamber gases were drawn off through the lower cock, while the upper one was open. Suction was furnished by the house vacuum system. Before the war gases were allowed to enter the vacuum system, they were passed through absorbents. For dimethylsulfate vapors 30 per cent NaOH wash bottles and soda lime tubes were used; for the chloropicrin gas, charcoal tubes were introduced into the system. The routine time allowed for evacuation was 30 minutes.

Thick rubber gloves were worn while removing the cage from the gas chamber and while cleaning it and the wire cage. The laboratory helpers were also encouraged to wear the regulation army gas mask (older form with nose clip) as a routine measure during the process of gassing; an hour's trial, however, invariably brought the conviction that this additional factor of safety was superfluous.

The precautions observed may seem excessive when it is considered that fractions of a cubic centimeter of the war liquids sufficed to give the desired gas concentration in the chamber, and that this amount completely vaporized in an average sized, well ventilated room would be entirely devoid of danger. The precautions, however, were made obligatory to counteract the depressing mental effect which war gas work exerted initially upon the helpers.

The methods employed in testing the analgesia produced by dimethylsulfate and by chloropicrin will be described in the section dealing with the experiments.

General Picture.

Before entering upon the subject of this paper it is necessary to give a brief description of the chief reactions which the cat exhibits during and after the period of gassing. While being gassed with dimethylsulfate in a concentration of 3 to

5 per 10,000 of air, the cats show a swiftly increasing irritation of the respiratory mucous membranes and of the conjunctiva. There is, first, winking of the eyelids, narrowing of the lid aperture, licking of the nose, and increased tear secretion. A little later the eyes are kept more or less closed. Sneezes and slight coughs now are noticed and a clear, watery saliva drips from the jaws. This watery saliva is soon replaced by a viscous secretion which hangs in gelatinous ropes from the angles of the mouth. At this stage the mouth is kept open most of the time, as apparently the nasal passages are obstructed. The respiration of the cat varies from 20 to 27 per minute and is not labored; towards the end of the gassing period most animals show short spells of polypnea, during which the tongue is protruded and the cat pants like a dog. Vomiting may occur during the last half of the gassing period or shortly after removal from the gas chamber. In general there is only a moderate restlessness at the start and then the animal lies or squats in a normal position on the floor of the cage. Certain animals, however, exhibit considerable excitement while being gassed, and this was more frequent among females than among males. The excitable cats were generally more severely affected by the same concentration than their more stolid mates. The mucous membranes of the mouth, and the tongue were usually red-pink throughout the gassing period, only occasionally was a slight cyanosis observable. It is worthy of note that most of the cats seemed astonishingly comfortable on the whole in the gas chamber.

After removal from the gas chamber the cats appeared to be in excellent general condition, but were disinclined to make any unnecessary movement; some muscular impairment was shown occasionally by a slight stagger on walking and by the short duration and weakness of resistance when examined. The animals preferred to crouch quietly in one place with head slightly extended and eyes closed. Inspection showed the jaws slightly open; slight or no salivation; moderately slow respiration, about 25 or 30 per minute, accompanied by rhonchi; expiration was slightly prolonged and active. On gently separating the lids the corneas generally exhibited a centrally located, oval, milky opacity, and the conjunctivæ and nictitating membranes were reddened and swollen. Auscultation revealed the lungs filled with snoring, groaning, clucking râles. These râles largely obscured the heart sounds. The blood pressure in the femoral artery was always at least 100 mm. Hg., generally more. From now on the course was usually progressively downward. The respiration became more and more labored; inspiration was slow and accompanied by a wide opening of the jaws; occasionally some retraction of the costal margins was observable during inspiration; expiration was usually prolonged and active. The mucous membranes were usually of a good color, but slight exertion of the animal sufficed to bring on a definite cyanosis. Death generally took place within 4 days, though some cats survived for months.

No food or water was taken by the cats which succumbed in 3 to 4 days; any attempt to swallow apparently led to choking.

Autopsy revealed a marked inflammation of the entire respiratory tract. The pharynx was reddened and occasionally covered with patches of yellow exudate;

the epiglottis was reddened, edematous, and occasionally covered with a gelatinous exudate. In some instances the tip of the epiglottis was occupied by a vesicle of edematous tissue. The entrance to the larynx may be a mere slit in a water-logged mass of tissue.

On opening the trachea and bronchi the entire mucosa was found covered by a grayish, gelatinous, thick membrane which extended apparently into the finer bronchioles. This membrane could be readily removed and formed a tough mass; frequently it was possible to obtain what seemed to be perfect tubular casts of a large part of the bronchial tree. The thickness of this pseudodiphtheritic membrane was often so great that the tracheal lumen on inspection seemed largely occluded, and some of the smaller bronchi were apparently completely plugged. Pulmonary edema was not frequent, and when present was remarkably localized, involving only one lobe or only a portion of a lobe.[2]

Chloropicrin gas in a concentration of 1 to 10,000 for 30 minutes or 1 hour causes symptoms which are in general similar to those obtained with dimethyl-sulfate. The main differences will appear in the following brief description.

As soon as the cat or cats are introduced into the gas chamber and before the chloropicrin has been heated, the animals wink rapidly and salivation begins. Within 1 minute ropy masses of saliva hang from the angles of the jaws; oftentimes clear, watery saliva drips over these glairy appendages. The salivation reaches a maximum and towards the end of the gassing period may have largely disappeared, as in dimethylsulfate intoxication. Retching and vomiting may occur early and is more frequent than with dimethylsulfate. During the early period of gassing a moderate restlessness is apparent. This is followed by a stage of quiescence during which the cat squats or crouches in the cage. Occasionally a short fit of restlessness intervenes. The respiration at the beginning of gassing is about 25 per minute; towards the end of the period it is generally shallower and faster, 50 per minute, with a slight active expiration. The prolonged active expiration seen with dimethylsulfate was absent, nor was opening of the jaws with inspiration such a prominent feature during and after chloropicrin gassing as when dimethyl-sulfate was employed. There was no evidence of marked asphyxia throughout the experiment, judging from the pink color of the tongue and mucous membranes of the mouth.

After removal from the gas chamber the cats were quiet and only slightly salivated. The respiration often was composed of short, weak inspirations followed by a sudden, sharp, active expiration. Auscultation showed the chest filled with coarse râles, which obscured the heart sounds. Muscular weakness was shown by feeble resistance to extension and by a disinclination to move. In the majority of instances death occurred within 48 hours after gassing with chloropicrin 1 : 10,000 for ½ to 1 hour; a few cats, however, survived for months.

The autopsy exhibited a picture quite different from that produced by dimethyl-sulfate. The larynx and trachea were moist and pale, without any congestion

[2] Auer, J., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 106.

or membrane formation. Foam and fluid were generally found in the trachea; the lungs were large, heavy, and mottled with moist brownish areas and fresh hemorrhages. On closer examination these brown areas were seen to be areas of lung alveoli completely filled with fluid; the borders of the lobes were often emphysematous. On section, fluid poured from the cut surface as if from a saturated sponge. The parenchyma also often revealed numerous and large hemorrhages. On the surface of the section each bronchus and bronchiole was surrounded by a translucent, moist, gray zone of edema and the lymphatic trunks near the hilus were always engorged with clear fluid. This engorgement of the pulmonary lymphatics is observable at a time when the lung alveoli contain but traces of edema fluid, for example in cats killed after 15 minutes exposure to the gas. The edema was usually most pronounced in the lower lobes, though all lobes were involved. The degree of edema was often so great that moderate compression of the chest caused a gush of liquid from the trachea.

The diaphragm level was often abnormally low, due to a tonic contraction of its muscle fibers as indicated by the ridge formation of the muscle tissue at its tendinous insertion. This tonic contraction was also observed in cats in which a biopsy was carried out, and is perhaps ascribable to an *intra vitam*, partial rigor.

The heart muscle was usually brownish in color and flabby. On section, macroscopic hemorrhages in the left branches of the His bundle were frequently observed.

Analgesia.

Reduction of the capacity to perceive ordinarily painful stimuli was first observed during a tracheotomy performed to save an animal from the effects of a laryngeal stenosis. This animal had been gassed with dimethylsulfate 24 to 36 hours previously when suddenly it showed restlessness, the tongue and mucous membranes became bluish, the respiration was labored, and with each inspiration the costal margins now sank inwards. Tracheotomy was carried out at once and the symptoms were relieved.

Before describing the tests it is important to emphasize that all the manipulations were carried out with the greatest care and were always executed in a fixed way. The mild tests were invariably applied first and if they indicated analgesia, other procedures were cautiously attempted. As soon as a definite restlessness developed the test was discontinued. The cats were usually killed by injecting 10 to 15 cc. of ether into the trachea.

The tests were carried out while the cats were either lying or squatting on a table or when they were extended back down. A sharp, brief pressure of the pinna of the ear, the nasal septum, lips, the

skin of the body, the toe pads, and tail by means of a hemostat caused in typical cases no sign of pain. After the stimulus, the compressed ear was flattened against the skull, the head drawn to one side, the foot pulled away, or the tail twitched aside. In the normal cat a sudden moderate pressure upon the tail usually calls forth a short, strident yowl, an arching of the back, and a sidewise retreat while the animal spits a few times and glares with apprehensive resentfulness at the experimenter.

With the greatest precaution further tests were then made. It was found that the carotid or femoral artery could be exposed and freed from the accompanying nerves, that large flaps of skin could be resected, and that the tibialis anticus muscle and tendon could be dissected free, without causing any or only slight and indefinite evidences of pain. These evidences consisted chiefly of a slight restlessness and probably denoted discomfort rather than pain, for normal cats are not diffident about responding vocally to traumatic stimuli.

Still further experimentation showed that the peritoneal cavity could be opened in the midline; that, furthermore, a loop or loops of intestine could be delivered through the laparotomy wound and gently pulled, or that the parietal peritoneum could be rubbed with the finger without eliciting more than a slight general rolling motion of the body (Figs. 2 and 3).

However, marked stretching of a mixed nerve or of the mesentery, a strong electrical stimulation of an afferent nerve, all produced signs which could be interpreted as evidences of some perception of discomfort or even pain: the animal became moderately restless, the respiration quickened and deepened, and the blood pressure exhibited some elevation of its level.

The analgesia was generally well established within a few hours after gassing and within 24 hours reached a maximal degree. These animals were often in an apparently excellent condition, and Figs. 1 and 2 present an autographic record of how animals of this type fail to respond to ordinarily painful stimuli.

The record of Fig. 1 was furnished by a cat which had been gassed with dimethylsulfate in a concentration of 4.5 to 10,000 air for 1 hour. This animal was a male, orginally weighing 4,150 gm., and had been

fed shortly before gassing. 26 hours after gassing he was in good general condition, though respiration was slow, slightly audible, and accompanied by some retraction of the costal margins; expiration was active and prolonged; the respiration rate was 32 per minute; his voice was hoarse. There was no obvious muscular weakness and the animal jumped with ease and coordination from the examining table to the floor. No food had been taken since exposure to the war gas, and his weight now was 3,760 gm., showing a loss of 390 gm. As soon as the animal was extended upon his back, the inspiration became more labored and snoring, and retraction of the costal margins increased in extent. The trachea was now exposed and partially slit transversely; the dyspnea was at once relieved, the inspiration now was unlabored and no longer accompanied by a snoring sound or by a costal retraction; expiration was also easier but was still moderately active. The trachea was surrounded by a zone of emphysema and the tracheal lumen was partly obstructed by a thick, gelatinous, membranous sheath, a part of which was removed.[3] The operation developed not the faintest sign of pain; the animal was perfectly quiet and the tongue and mucous membranes had a better pink color than before. The left tibialis anticus muscle and left peroneal nerve were now completely dissected free and the nerve cut without any sign of pain; the cat rested quietly on its back and only the respiration showed a transitory acceleration in rate after section of the nerve. The animal was then prepared for recording the blood pressure in the right femoral artery. The section of the skin, the freeing of the artery from the crural nerve by blunt dissection, the placement of the nerve on a loop of thread, all caused no restlessness and the animal remained as quiet as if under the influence of an anesthetic. Stimulation of the intact crural nerve with the faradic current (300 mm. coil distance, two Daniell cells, Petzold coil, cable electrodes) induced a slight rolling motion of the animal accompanied by a blood pressure rise of 13 mm. Hg. (Fig. 1). Slight movements of the cat also caused elevations of the pressure by about 20 mm. Hg.

[3] Removal of the tracheal membranous exudate is always dangerous because a section may be aspirated and plug the trachea and bronchi. For the same reason a transverse section of the trachea should never completely sever the tube of membranous exudate.

Exposure and severance of the left vagosympathetic nerve were painless. The cardiac vagus endings exhibited perhaps some reduction of sensitiveness, for a coil distance of 100 mm. was necessary to obtain the characteristic slowing of the heart (Fig. 1). The motor vagus endings in the esophagus reacted vigorously when the peripheral vagus was electrically stimulated with the coils 150 mm. apart. Stimulation of the central vagosympathetic trunk produced characteristic respiratory effects: slowing and deepening of the respiration associated with inspiratory stoppage broken by short vigorous expirations when a strong current was used (150 mm. coil distance; see Fig. 1). No respiratory stoppage in expiration was observed with the current strengths employed. The blood pressure exhibited the characteristic depressor type of fall when the left central vagosympathetic trunk was stimulated. The depressor portion of the vagosympathetic trunk was exceedingly sensitive, mere placement of the nerve stump on the platinum electrodes sufficed to cause a fall of 20 mm. Hg. (Fig. 1). The eye responded well to stimulation of the left central vagosympathetic trunk; with 250 mm. coil distance the nictitating membrane retracted slowly, but the pupil showed no dilatation; with 200 mm. the nictitating membrane retracted well and the pupil dilated strongly; on stoppage of the stimulation, the pupil contracted promptly to its original slit-like form. The tibialis anticus muscle exhibited no sign of fatigue when stimulated indirectly (peroneal nerve) with short tetanizing currents, 150 mm. coil distance, at 2 second intervals for 6 minutes. After 1 hour the rectal temperature was 35.7°C.

The depth of the analgesia was still further tested by opening the peritoneal cavity in the median line. There was no evidence of pain and the cat remained practically immobile. Insertion of the finger into the peritoneal cavity and gentle friction of the parietal peritoneum caused no resistance. The arteries of the intestine were bright red, while the veins were quite dark in color. The animal was then killed.

Quite similar results were obtained when chloropicrin was employed. The following abstract of a typical protocol, together with Fig. 2 giving the blood pressure record, will demonstrate this fact.

This cat was a non-pregnant female weighing 3,750 gm. and had

been fed previously; chloropicrin in a concentration of 1:10,000 was employed for 30 minutes. 23 hours afterwards the cat weighed 3,180 gm. but was in apparently good condition, though the respiration was moderately labored, with slightly snoring inspiration, about 44 to the minute, and was accompanied by an expiratory grunt. The expiratory grunt only became audible some time after expiration had begun. The tongue and mucous membranes were slightly cyanotic. The ordinary pressure tests yielded the same result which has been described in the dimethylsulfate animal. In this animal the dorsal position also increased the respiratory difficulty: the tip of the sternum now exhibited a definite depression with each inspiration and a moderate restlessness appeared. Tracheotomy was carried out at once and a fair amount of brownish fluid and foam escaped from the tracheal wound, which was increased by moderate compression of the chest and elevation of the foot of the board. As soon as the trachea had been slit the restlessness of the animal ceased, and it remained quiet; the tongue and mucous membranes became red without any trace of cyanosis. Some respiratory stenosis still existed, however, for the sternum still showed a slight depression with each inspiration. The right femoral artery and the left vagosympathetic trunk were also exposed. Fig. 2 shows the blood pressure record of this animal before, during, and after stimulation of the left peripheral and central vagosympathetic stumps; also before and after opening the peritoneal cavity and while gently rubbing the parietal peritoneum. The blood pressure curve of this animal reveals no significant changes while the parietal peritoneum was rubbed for approximately 10 seconds by the finger. The cat did not even move, for every movement is betrayed by a rise of pressure. The response of the cardiac vagus to electrical stimulation occurred with a weaker current in this cat than in the preceding animal; 200 mm. coil distance sufficed, while 100 mm. were necessary in the previous cat before a cardiac slowing was noticeable. The reflex respiratory effects in the chloropicrin cat after stimulation of the central vagus showed active expiratory stoppages and not inspiratory stoppages as in the dimethylsulfate animal. During these respiratory stoppages in active expiration foam and fluid were occasionally expelled from the trachea. The depressor nerve was not as sensitive in this animal as in the dimethyl-

sulfate cat. On the other hand, the chloropicrin cat exhibited a much greater sensitiveness of the dilator fibers of the cervical sympathetic, for a coil distance of 400 mm. sufficed to give a definite pupillary dilatation, while 250 mm. was the weakest current which was effective in the dimethylsulfate cat.

In this connection it must be mentioned that this cat exhibited definite hippus before tracheotomy had been carried out; the pupils dilated during inspiration and contracted well during expiration. The pupils responded to light and varied in diameter as the animal looked around (accommodation). The lid reflex was prompt. The arteries of the intestine were red, while the veins were moderately dark. The animal was killed later.

Autopsy of this cat showed a strong pulmonary edema; the lower lobes were full of fluid while the upper lobes contained less fluid but more foam and air.

These two abstracts and tracings (Figs. 1 and 2) reveal unmistakably that the capacity for pain perception, both superficial and deep, may be enormously reduced by exposure to the two war gases mentioned. The degree of analgesia exhibited by the two cats described above was not observed in all animals but in the great majority the presence of a well defined, undoubted analgesia could be readily established.

The duration of the analgesia could not be determined with accuracy because most of the cats succumbed within 4 days. After chloropicrin intoxication a number of cats exhibited, about 5 days after gassing, outspoken signs of pain perception when mild pressure tests were applied to the tail. The reaction was so definite that no further tests were made. The same reestablishment of pain perception was also noted in a chloropicrin cat which survived for 5 months. After dimethylsulfate, on the other hand, the analgesia may still be present to a remarkable degree even after the lapse of 6 months. Fig. 3 illustrates the one instance I have observed.

This cat was a male weighing 4,250 gm. originally, and had been subjected to the effects of 4 parts of dimethylsulfate to 10,000 of air for 1 hour. The effects were severe and the animal did not show a change for the better until 7 days after gassing. 10 days after gassing, food was taken more regularly and the animal moved about in a practically normal fashion. 6 months after gassing, the animal

was still alive though markedly emaciated, and weighed 2,890 gm., which represents a loss of 1,060 gm. The emaciation was not due to failure to eat, for the animal ate heartily. There was no definite muscular weakness discernible and he walked and jumped on occasion like a normal animal. The nose showed a yellowish fluid discharge. Both corneas were spotted with a central staphylomatous leucoma and synechias stretched between the upper pole of the iris and the cornea of each eye. The respiration was slow and easy, with slightly prolonged expiration, and the rate was 40 per minute. Moists throaty sounds often accompanied the respiration. The usual test, were carried out and no definite signs of pain perception were elicited. Fig. 3 represents the blood pressure curve of the animal while being tested for deep pain perception. Both vagosympathetic trunks were intact and no tracheotomy was performed in this cat. It will be observed that a pull on the crural nerve caused only a slight short rise of pressure which was considerably less than that obtained when the animal was slightly restless just preceding this test. Opening of the abdominal cavity caused no change in the pressure level worth mentioning nor did restlessness occur. That the procedure had caused reflexly effective afferent impulses is seen, however, in the rise of the pulse rate from 140 to 160 and in the increase of the respiratory rate (25 to 32 per minute). Introduction of the finger into the abdomen caused a short rise of the blood pressure from 126 to 148 mm. Hg. and was associated with slight general movements. These reactions surely indicate that sensory impulses reached the higher centers, but from the general behavior of the cat it seems equally certain that these sensory impulses were perceived as uncomfortable sensations rather than as pain sensations. The cat was then killed by means of ether.

DISCUSSION.

The observations reported on preceding pages indicate clearly that cats gassed with chloropicrin or dimethylsulfate under the conditions mentioned do not react normally when subjected to stimuli which are painful under ordinary conditions; operative interferences may be undertaken, involving the skin, the deeper interfascial structures of the leg and neck, or the peritoneum even may be incised and its

parietal leaf rubbed, without eliciting the ordinary signs of pain. In other words, gassing cats with one of these two war liquids endows the treated animals with a marked degree of analgesia both for superficial as well as for deep pain stimuli.

The question now arises how this experimental analgesia is produced, whether there is any single factor at work which may explain this curious effect of at least two, chemically different war gases. There are four possibilities to be considered: (1) anesthesia of the sensory nerve endings of the skin; (2) blocking of sensory impulses in the nerve trunks; (3) that the analgesia does not exist in reality but is simulated by the fact that motor weakess prevents expression of the normal reaction to pain stimuli; and (4) that the higher centers are largely unable to perceive pain stimuli. It must be stated at once that some of these factors can be excluded with considerable certainty, yet perhaps all may be involved to some degree in the causation of the final result; on the whole, however, one factor appears to play a dominant rôle. The various possibilities will now be discussed briefly.

It might be assumed that the receptive sensory endings in the skin are damaged by the war gas vapors which condense to some extent at least in the fur of the animals. Such a condensation apparently does take place, for the behavior of fleas, with which most of the cats were infested, indicates this. Shortly after gassing the fleas always became restless, left the deeper portions of the fur, and climbed to the extreme tip of the longer hairs where they maintained a precarious position with difficulty until increasing weakness caused them to fall or jump feebly to the floor of the gas chamber where they died. The experimental evidence at my disposal does not permit any statement as to whether or not the two war gases produced the postulated skin anesthesia, but the facts already submitted show definitely that such an anesthesia fails to explain the presence of analgesia to deep pain stimuli (stimulation of nerve trunks, opening of the peritoneal cavity; see Figs 1 to 3). The localized analgesia observed in gassed soldiers by Naiding would seem to indicate, however, that under certain conditions some war gases may destroy the functional activity of the sensory skin endings. It is possible that this local action was reinforced by a systemic effect, for Naiding states that

80 per cent of his patients complained of pain in the chest, asthma, or coughing.

The second possibility was a blocking of sensory impulses in the nerve trunks subsequent to the inhalation of the war gases. The experiments and tracings give no support to this supposition. In numerous instances typical analgesia was present when examination revealed a prompt knee jerk, a pupil responding normally to light, accommodation and sympathetic nerve stimulation, and prompt, effective, medullary reflexes when the central vagus and depressor nerves were stimulated (see Figs. 1 and 2). These results would be impossible if conductivity in the nerve trunks were strongly reduced. Whether or not, however, a qualitative blocking for pain stimuli existed cannot be discussed profitably in the present state of our knowledge.

The third possibility considered the analgesia as non-existent in reality and assumed that peripheral motor weakness prevented the normal response to pain stimuli. This explanation is absolutely disproved by the fact that stimulation of a motor nerve produces normal muscular contractions, and by the further fact that the gassed muscle, for example the tibialis anticus, may show little if any greater fatigability than a normal muscle when stimulated directly, or indirectly through the severed peroneal nerve, the muscle being left *in situ* with blood and motor nerve supply intact.

There remains for consideration the fourth possibility, that the higher centers are largely unable to perceive pain stimuli as such, and this assumption satisfactorily explains all the observed facts. The foundation for this belief has already been presented by exclusion in the discussion of the three explanations of war gas analgesia, all of which placed the cause in the periphery. The dismissal of these peripheral causes as inadequate or inadmissible compels the view that the cause must be located in the higher centers. The higher cerebral centers, therefore, after gassing with dimethylsulfate or chloropicrin are apparently so altered that ordinary pain stimuli are no longer recognized as such, and this alteration does not appreciably interfere with execution of most of the basic reflexes which link the functional organism into a unit.

What the nature of this alteration may be can be inferred to some

extent after determination of the active agent which causes these specific changes in the higher nerve cells. In the search for this agent it might be assumed that the war gases producing analgesia perhaps show similar chemical groupings or structures and that this is causally related to the production of analgesia. There is no definite basis for this supposition beyond the fact that the three gases which have been described as producing analgesia, dimethylsulfate ($(CH_3)_2 SO_4$), chloropicrin ($CCl_3 NO_2$), and phosgene ($COCl_2$), may all be considered methane derivatives. The observation, however, that generalized analgesia may persist for 6 months after gassing (see Fig. 3) renders the view quite improbable that the war gas itself is the agent directly producing the analgesia. As far as I know there is no volatile chemical substance, excluding sublimation of the heavy metals, which can exert an effect in the body lasting for months after a single administration. It is therefore necessary to seek among the secondary effects of war gases for the common agent which produces analgesia. This common agent I believe to be a general, persistent, low grade asphyxia. The reasons are as follows:

It was noted that moderate exertion of short duration changed the pinkish buccal mucous membranes of the resting cat to a bluish cyanotic color; a mere change of position, for example extension on the back, often sufficed to bring on a notable cyanosis associated with restlessness, dilatation of the pupil and increased dyspnea; all of these undesirable results generally could be relieved by tracheotomy. Autopsy of these animals revealed the pulmonary air passages largely filled with a membranous exudate or with edema fluid. It therefore seemed clear that during life the lung ventilation barely sufficed for the vital needs of the organism, and that a small additional respiratory difficulty or increased production of CO_2 (struggles) brought on an obvious asphyxia. Furthermore, there seemed to be a definite association between the presence of marked analgesia and the functional capacity of the lung. Thus a number of chloropicrin survivors exhibited normal reactions to moderate pressure stimuli about 6 days after gassing. These responses were so clear that further tests were not deemed justifiable and were therefore omitted. After killing these cats with chloroform, examination showed the lungs to be in good functional condition; the color was pink, they

collapsed normally on opening the chest cavity, section revealed no trace of edema, and there was no stenosis of the air passages. The only marked abnormality was atelectasis of one or more of the upper lobes. In these cats, therefore, pulmonary ventilation of the blood was practically normal and no asphyxia existed. On the other hand, a dimethylsulfate survivor exhibited a typical analgesia 6 months after the date of gassing (see Fig. 3). Autopsy of this cat exposed a voluminous lung which collapsed but slightly on opening the chest; its borders were emphysematous and section disclosed the pulmonary passages including the trachea, apparently completely filled with a yellowish, semifluid, gelatinous exudate resting on a tough membranous base. The mechanical obstruction in the trachea, bronchi, and bronchioles was such that pulmonic ventilation seemed impossible, and yet no signs of respiratory distress were observed while the cat was walking about; indeed the cat seemed quite comfortable for it often raised its tail to a vertical position, rubbed itself against the observer, and made moist, purring sounds. In this cat a low grade, chronic asphyxia surely existed.

The various facts presented above legitimize the inference that a systemic, low grade asphyxia of pulmonic origin is a primary factor in creating and maintaining the generalized analgesia described in this paper. Other factors probably also contribute to the final result. Among these, inspissation of the blood[4] due to edema, salivation, vomiting, and deficient fluid intake, and cellular changes[5] in various parenchymatous organs of primary or secondary origin, may be of some value. The formation of methemoglobin apparently does not take place to any extent with the doses of dimethylsulfate and chloropicrin employed in these experiments. The most asphyctic blood readily turned to a bright red color when lightly shaken with some Ringer solution; moreover, Gildemeister and Heubner[6] report

[4] A careful study of various blood changes after chloropicrin, phosgene, and chlorine inhalations in the dog has been made by Underhill (Underhill, F. P., The lethal war gases: physiology and experimental treatment, New Haven, 1920).

[5] See Gildemeister, M., and Heubner, W., *Z. ges. exp. Med.*, 1921, xiii, 325, for a description of cellular changes after chloropicrin.

[6] Gildemeister, M., and Heubner, W., *Z. ges. exp. Med.*, 1921, xiii, 315.

only doubtful spectroscopic signs of methemoglobin in the blood of cats, even when the doses of chloropicrin were so large that death resulted within 15 to 18 minutes.

The analgesia described in the preceding pages may be of some practical importance in the human subject. In gassed soldiers, for example, where surgical intervention is necessary, it is quite probable that little or perhaps even no general anesthetic may be necessary. This would be desirable because it removes, for some patients at least, the danger of a general anesthesia by means of ether or chloroform in a partially asphyctic individual. Another benefit would be a conservation of the immediately available supply of anesthetics, a factor of some importance for stations in the field of operations where transport facilities are never ideal.

SUMMARY.

Cats gassed with dimethylsulfate or chloropicrin in such concentration that death generally results within 4 days, usually exhibit a marked generalized analgesia, both superficial and deep.

Gassed cats react with no obvious sign of pain to operative interferences, including laparotomy and gentle friction of the parietal peritoneum.

The analgesia develops within a few hours after gassing, and reaches its maximum in about 24 hours. With dimethylsulfate the analgesia may persist for 6 months; with chloropicrin practically normal sensitiveness has been observed 7 days after gassing.

This analgesia is considered to be caused and maintained largely by a general, low grade, tissue asphyxia which is chiefly of pulmonic origin.

EXPLANATION OF PLATES.

The blood pressure curves were recorded from the right femoral artery with a mercury manometer. The anticoagulant for the tubing was one-half saturated sodium sulfate solution.

The time marker registers 4 second intervals, and the time line represents also the line of zero pressure.

The broad white bands on the line below the time record indicate the time and duration of nerve stimulation.

l.p.v. indicates left peripheral vagosympathetic trunk; *l.c.v.*, left central vagosympathetic trunk.

Electrical stimulation: Petzold induction coil with two Daniell cells, platinum point cable electrodes; numbers refer to distance in millimeters between primary and secondary coils.

PLATE 4.

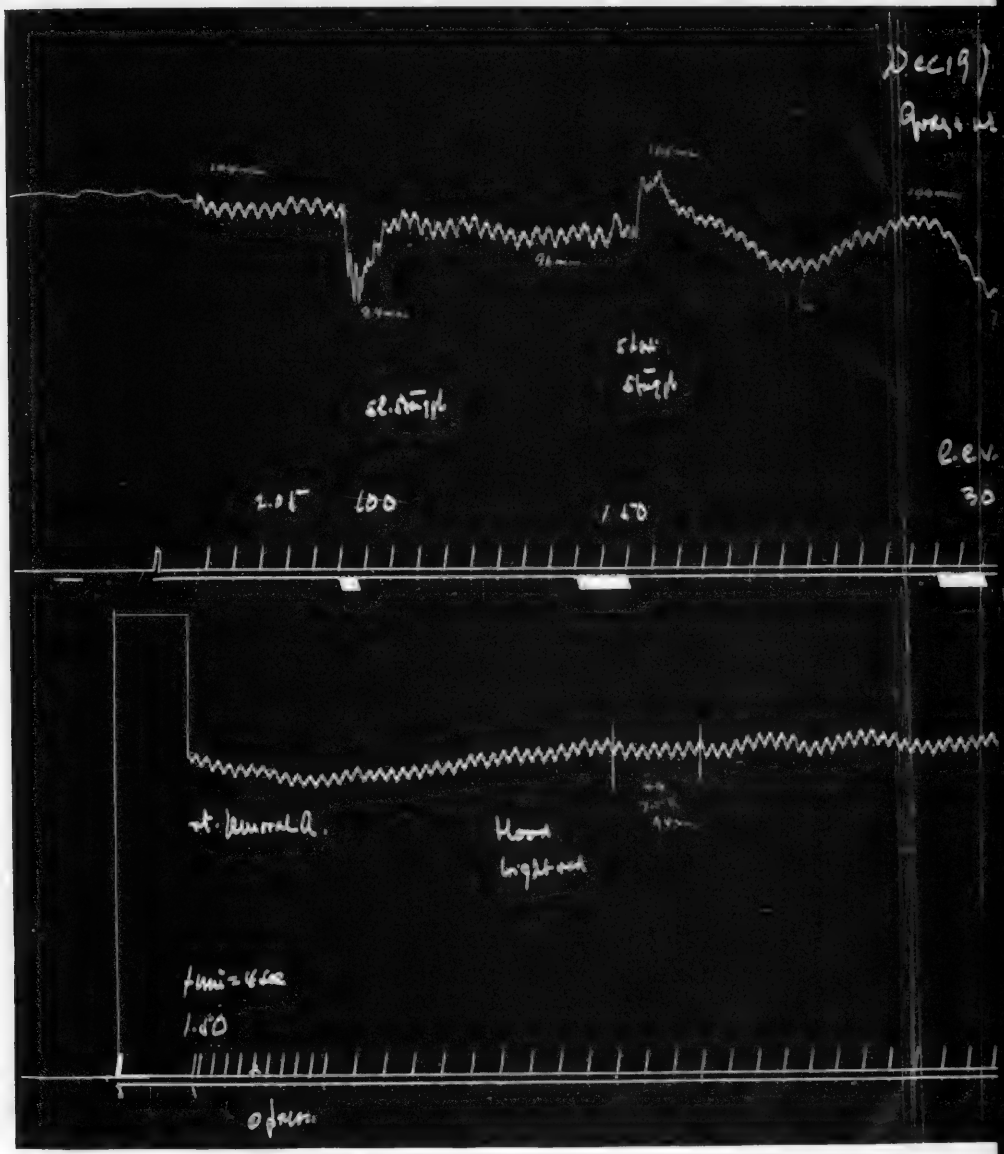
FIG. 1. Gray and white cat, male; weight 3,760 gm. Gassed with dimethylsulfate 4.5 to 10,000 for 1 hour. Tracing obtained 26 hours after gassing. Tracheotomy performed. Left vagosympathetic nerve cut during experiment; right intact. In this cat the abdominal cavity was later opened and the parietal peritoneum gently rubbed without causing any pain.

PLATE 5.

FIG. 2. Maltese cat, female; weight 3,180 gm. Gassed with chloropicrin 1 to 10,000 for 30 minutes. Tracing made 23 hours after gassing. Tracheotomy necessary because of pulmonary edema. Left vagosympathetic nerve severed before record was taken. Slight rolling motions at the beginning of the record are betrayed by the two moderate pressure elevations near the beginning of the curve. Note very slight effect of rubbing the parietal peritoneum for 12 seconds towards the end of the tracing.

PLATE 6.

FIG. 3. White and gray cat, male; weight 4,250 gm. Gassed with dimethylsulfate 4 to 10,000 for 1 hour. Tracing obtained 6 months later; weight at this time 2,890 gm. Both vagi were intact; no tracheotomy was necessary. Opening of the peritoneal cavity and insertion of a finger caused only a slight general movement associated with a rise of 20 mm. in blood pressure. The blood pressure, pulse rate, and respiratory rate were the same at the end of the tracing as at the beginning: 130 mm., 165 heart beats, and 30 to 32 respirations per minute.


Dec 19
R.C.V.
30
1.60
Blood
tmin = 16cc
1.60

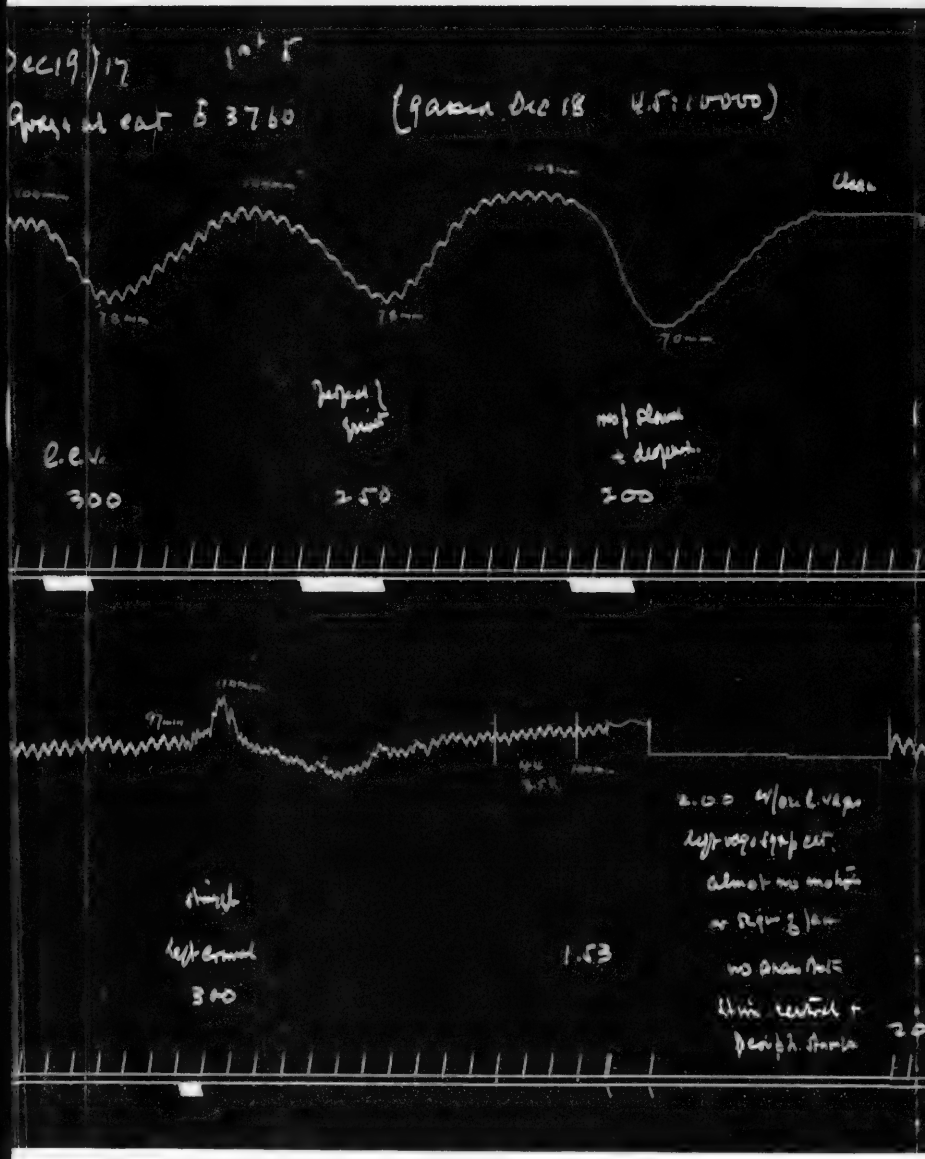
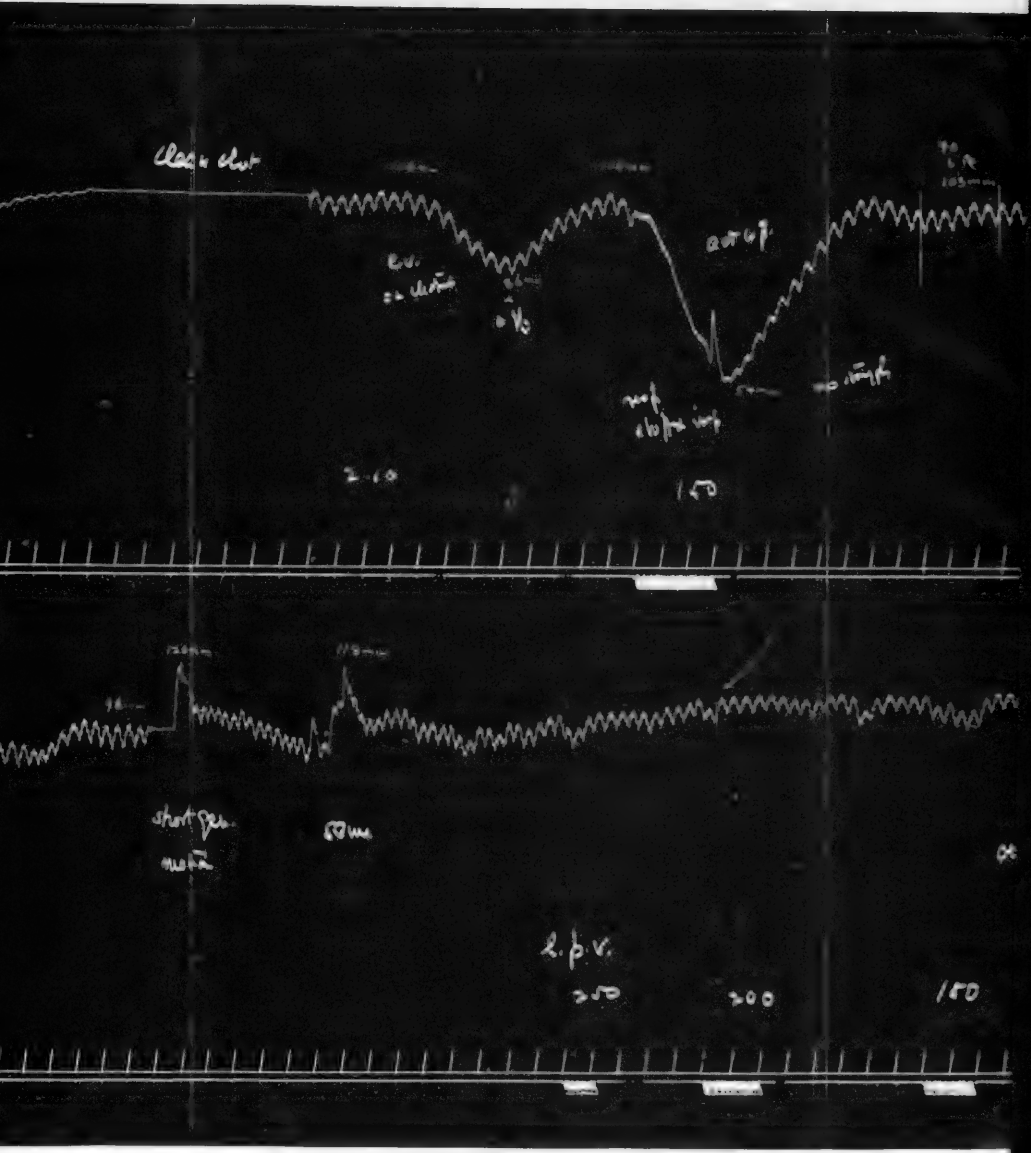


FIG. 1.


short pen.
50ms
2.p.v.
250
200
150

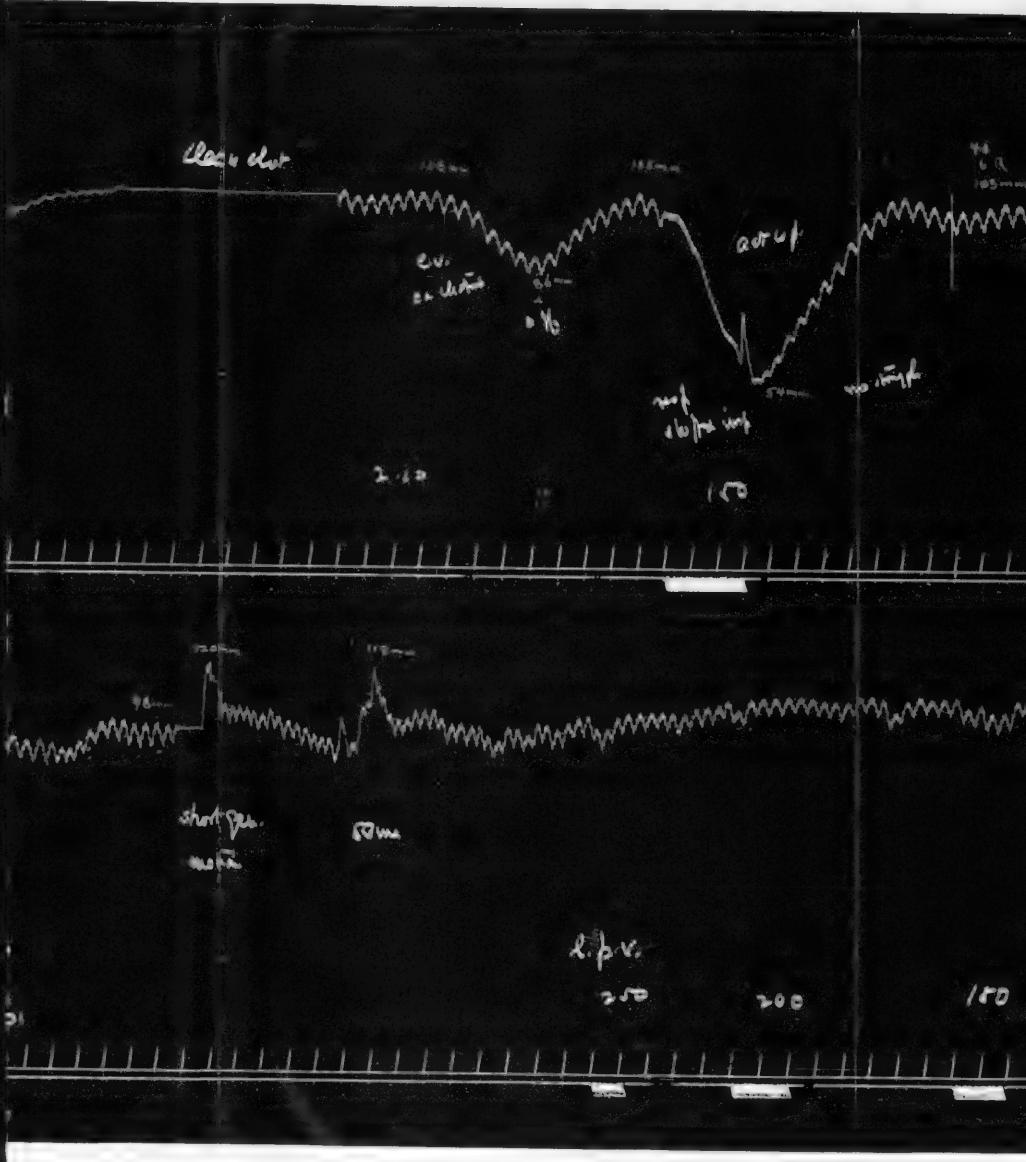
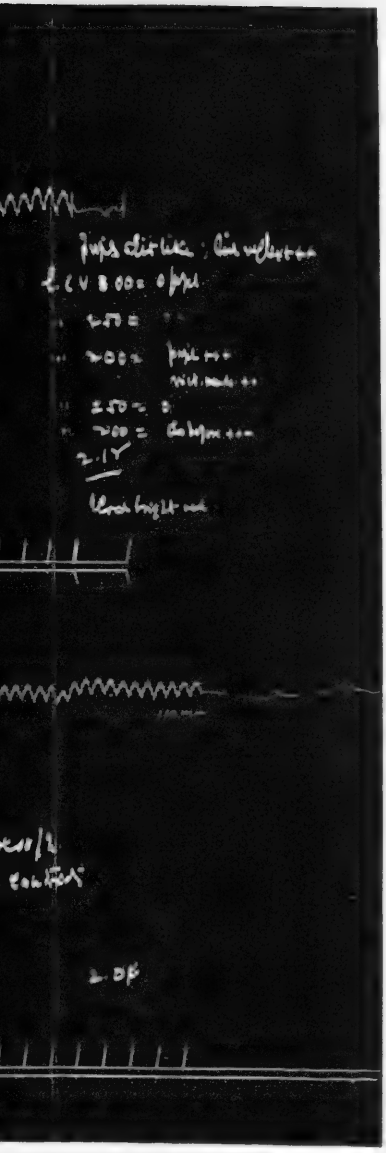
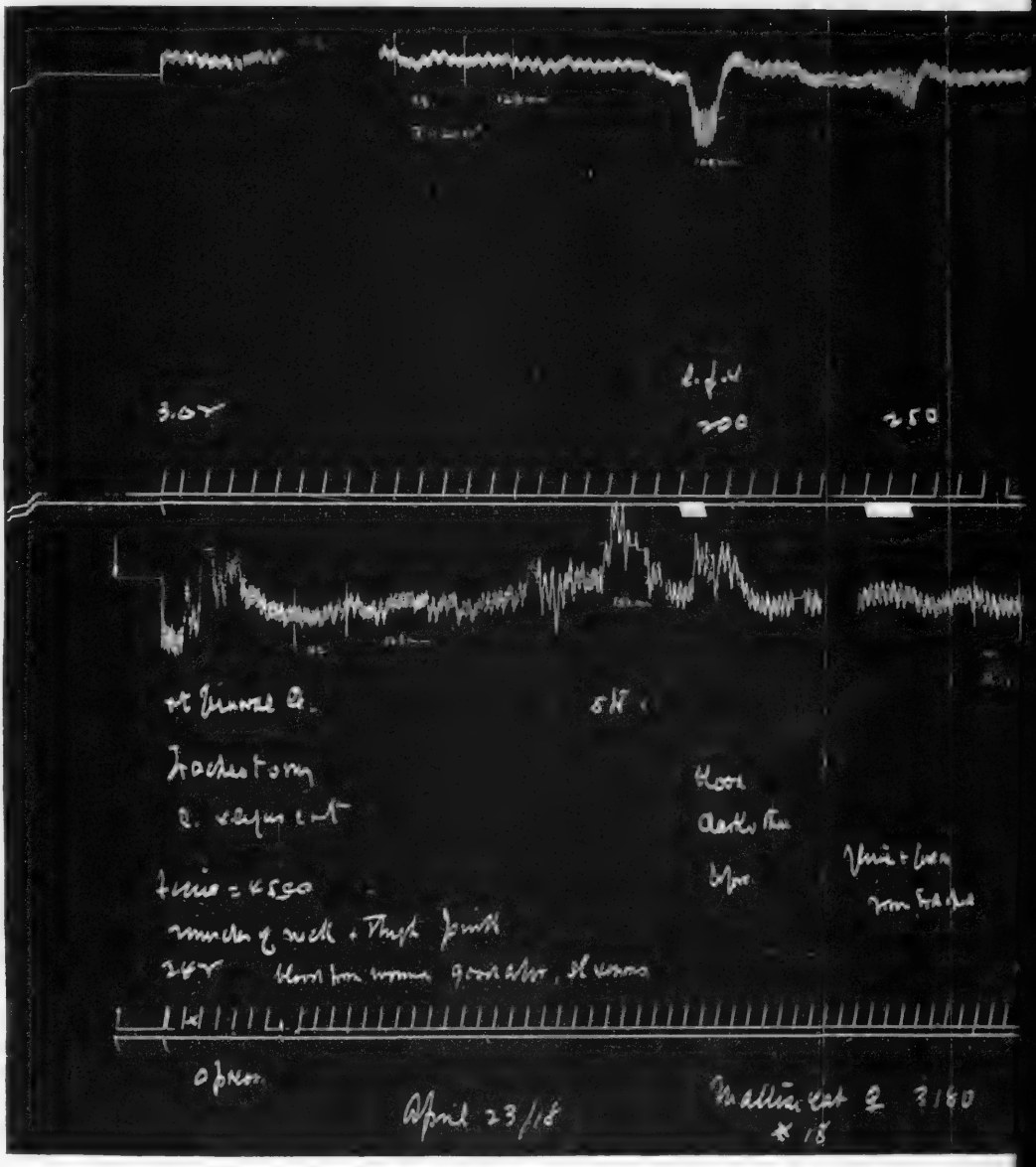

short pause
50 ms
200
150

PLATE 4.



(Auer: Analgesia after exposure to war gases)


3.0 V
250
Tracheotomy
Blood
Ureter + Ureter
April 23/18
Mallius cat ♀ 3160
* 18

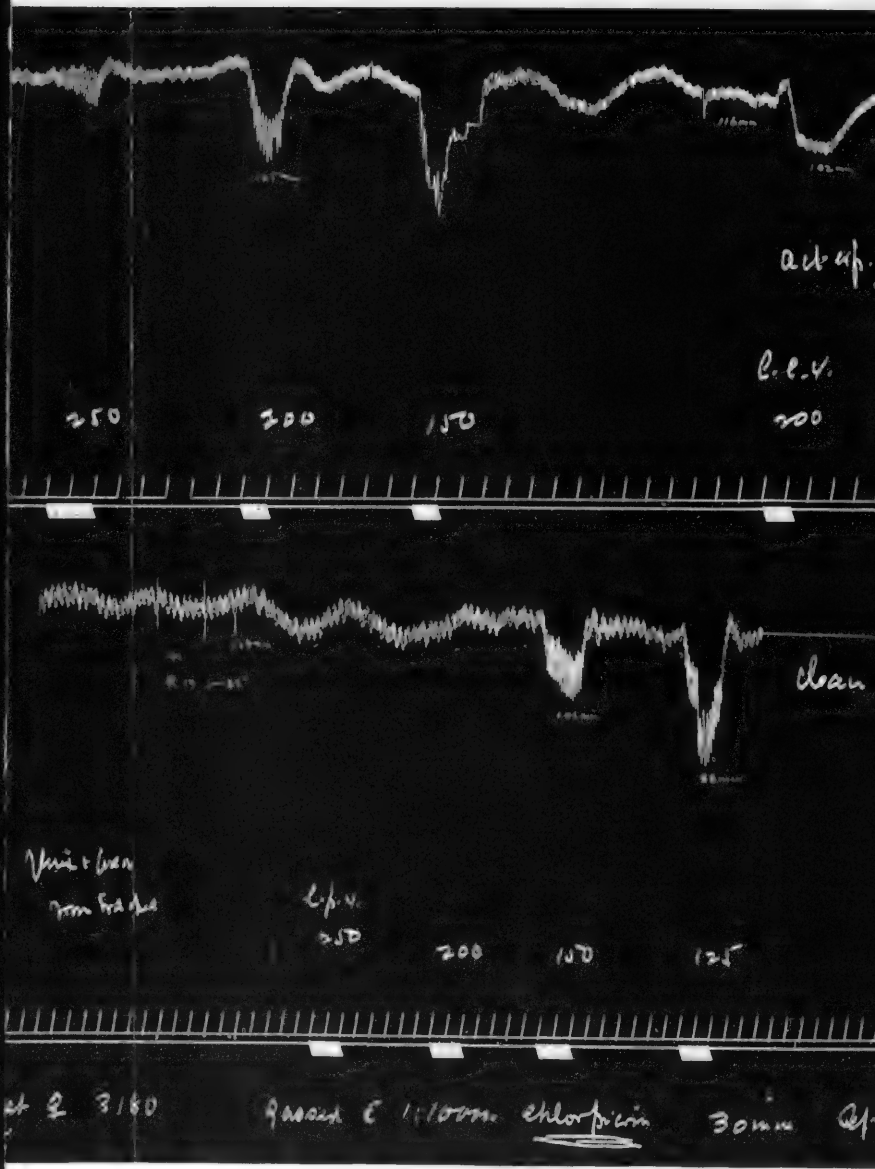
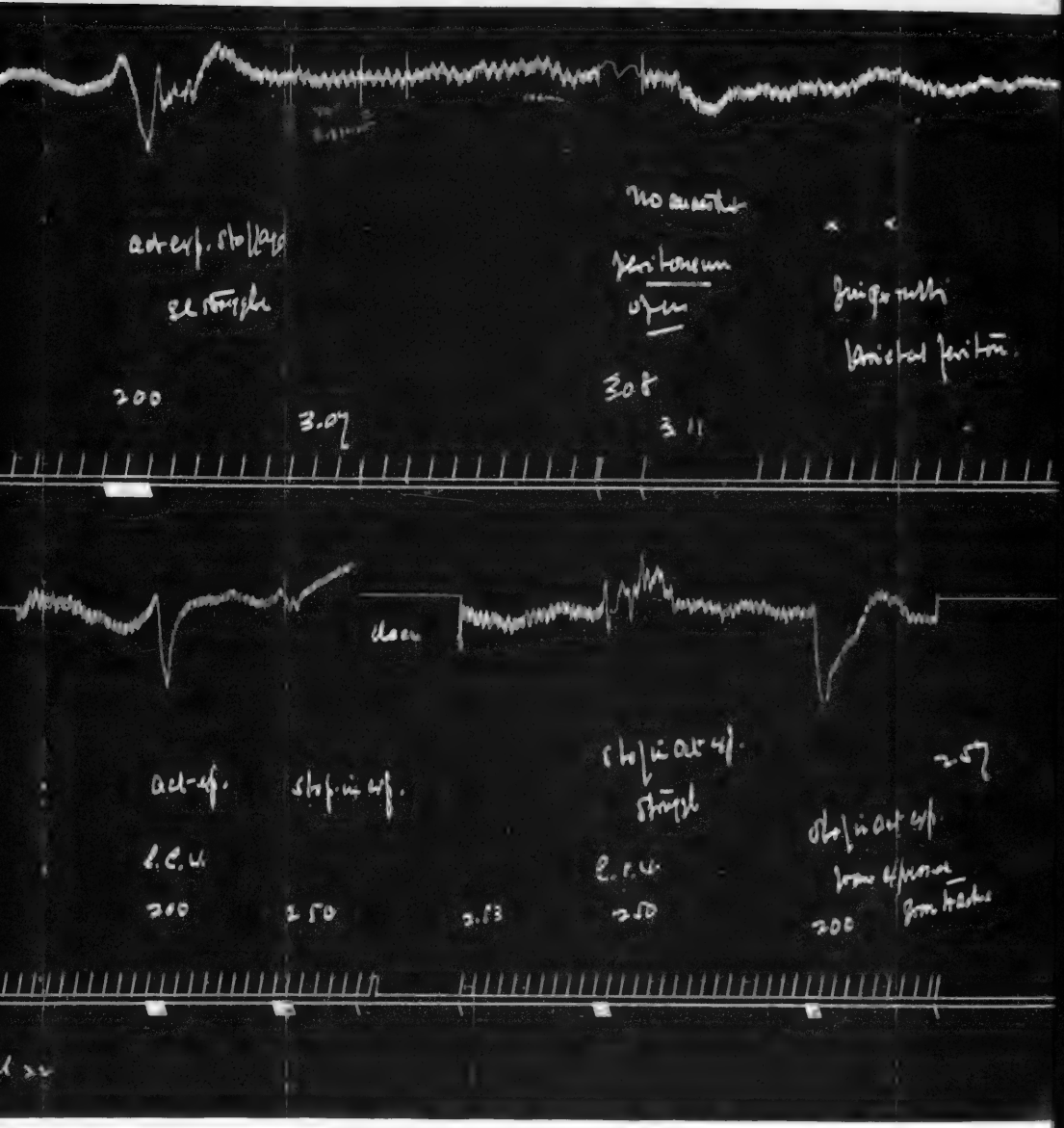
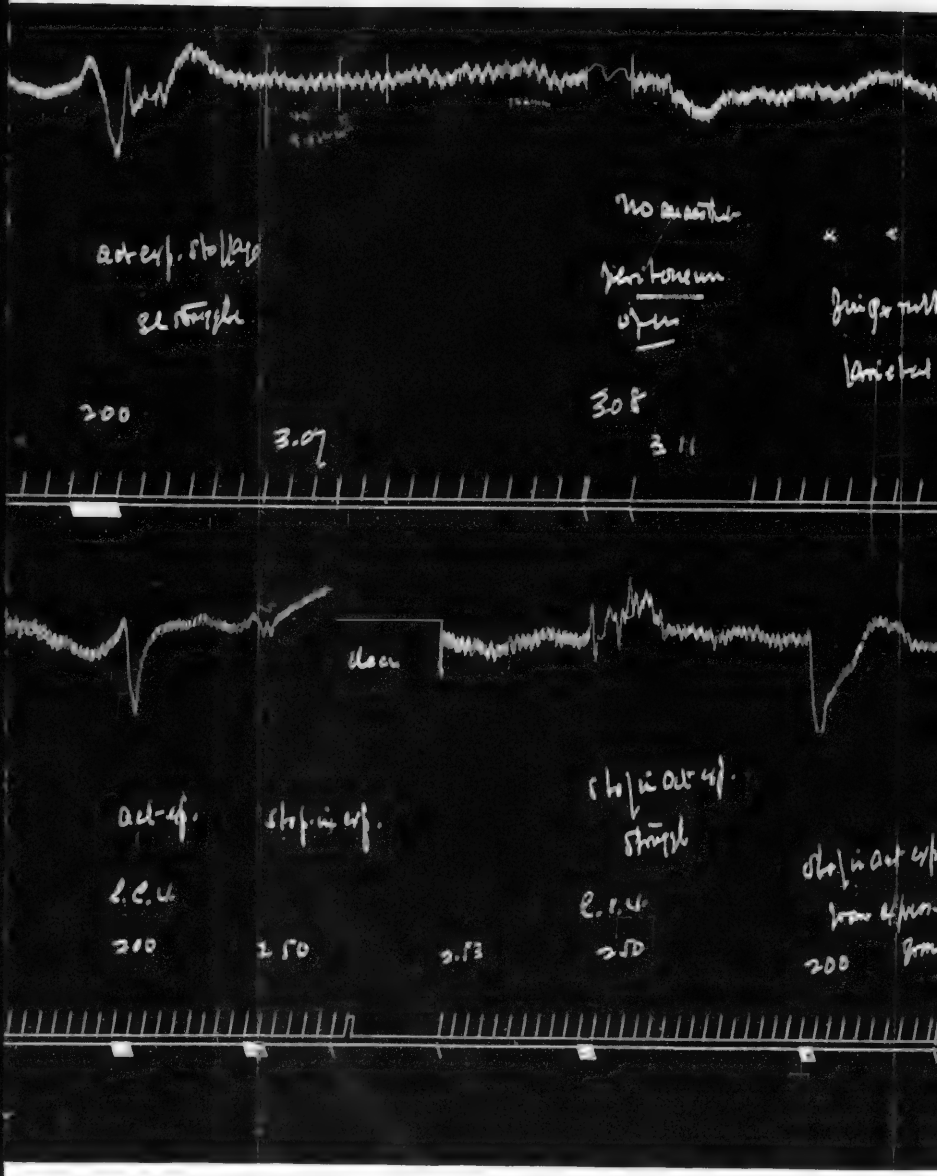
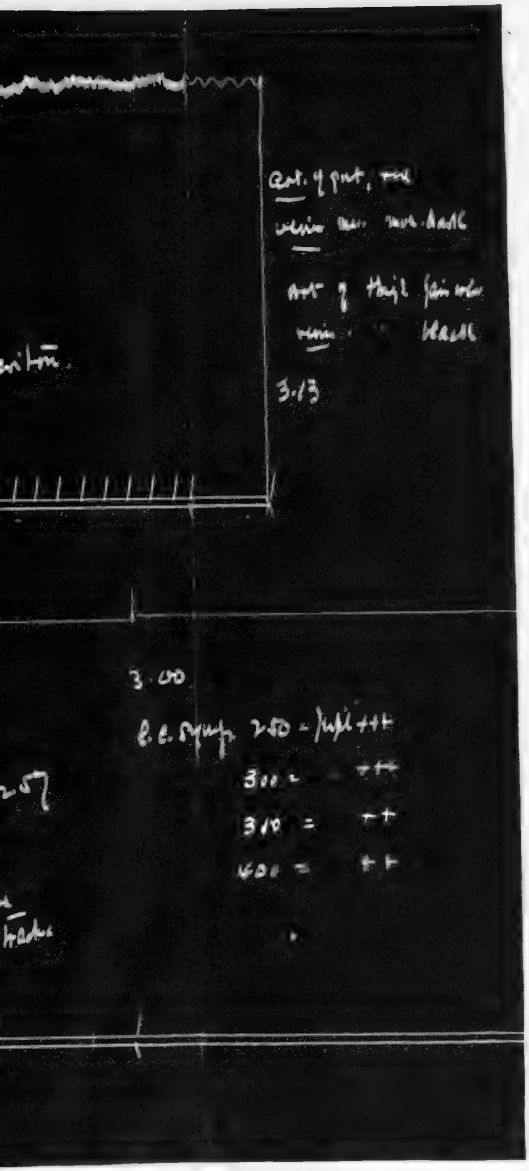


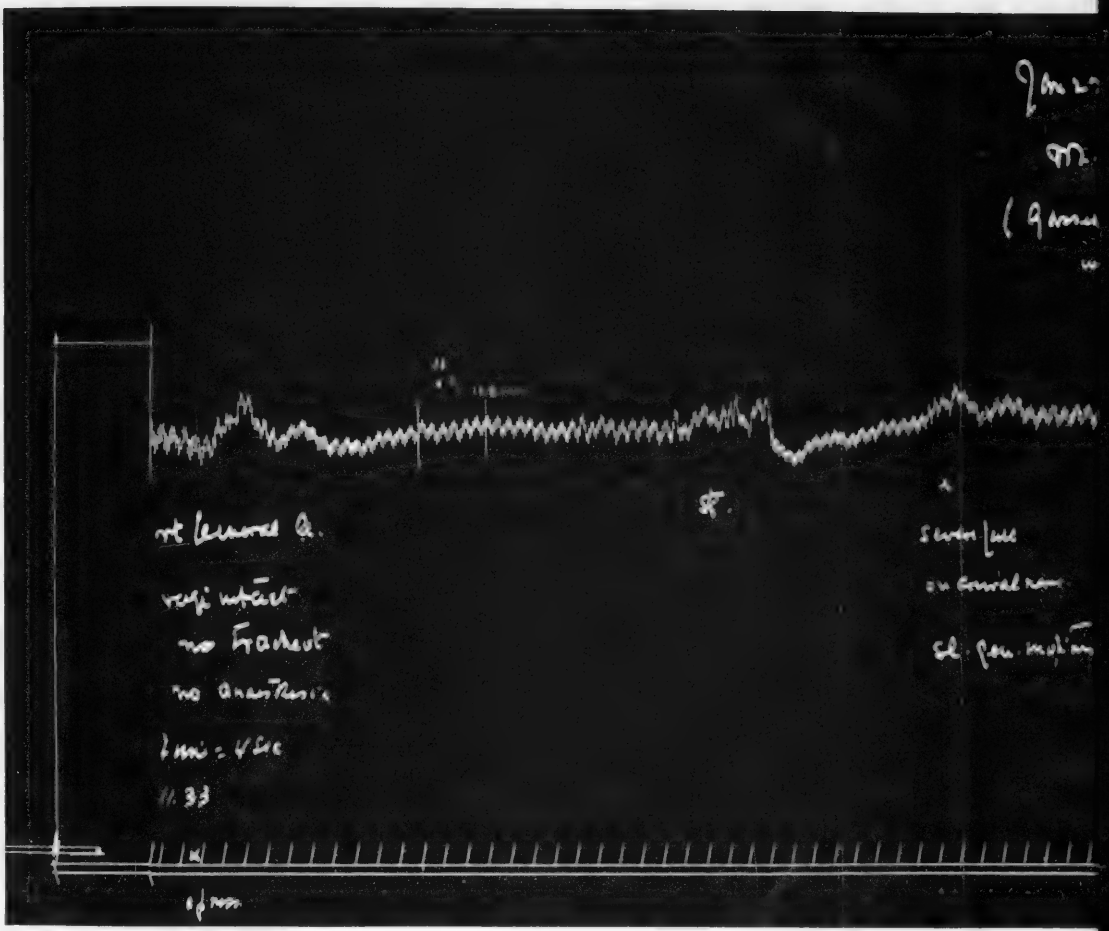
FIG. 2.


200
3.07
3.08
3.11
2.57
2.50
2.13
2.50
200


200
3.07
308
3 11
Aut-cyl. Stop/Start
El Stimulus
No reaction
Peritoneum
open
Stop
Aut-cyl.
Stop in aut cyl.
Stop in aut cyl.
Stimulus
Stop in aut cyl
200
2 50
2.83
250
200



(Auer: Analgesia after exposure to war gases.)



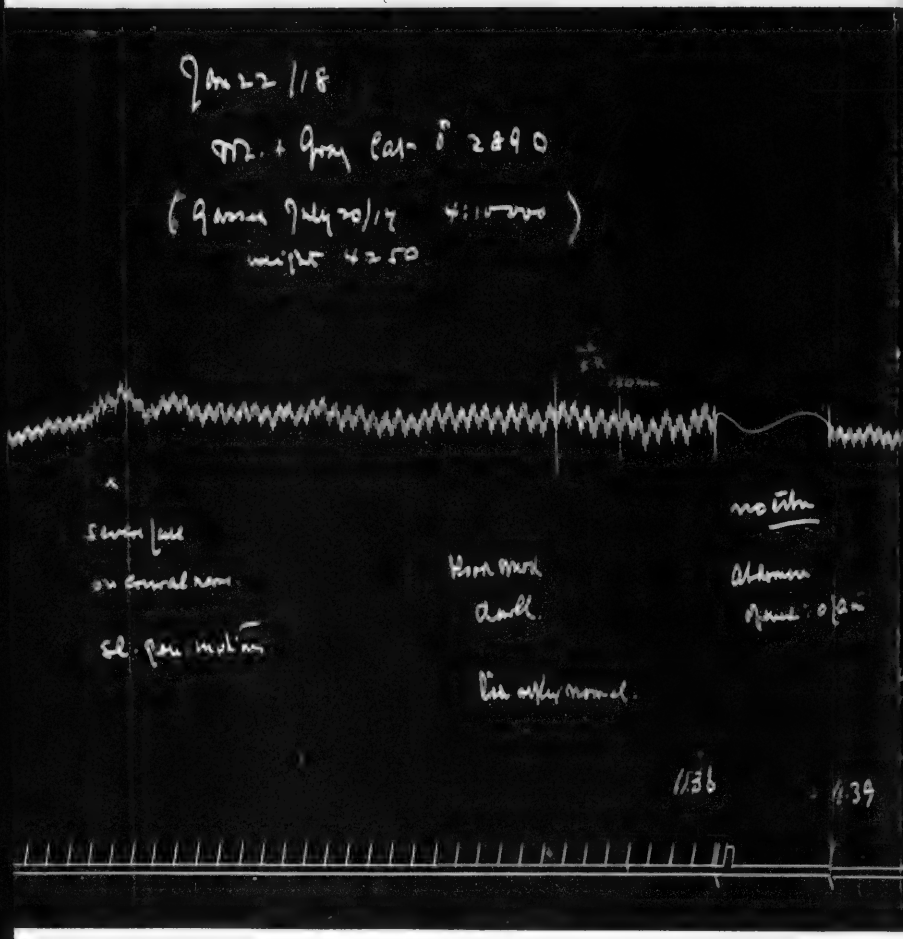
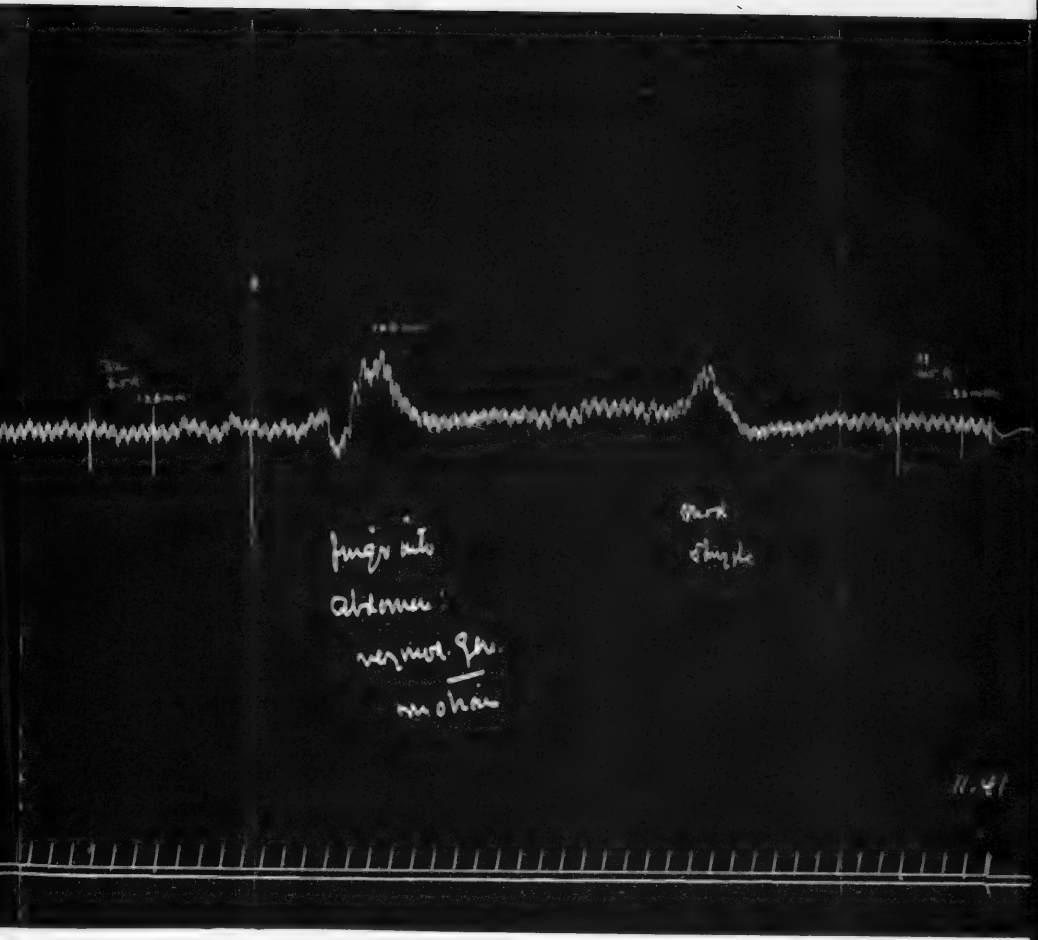


FIG. 3.



(Auer: Analgesia after e

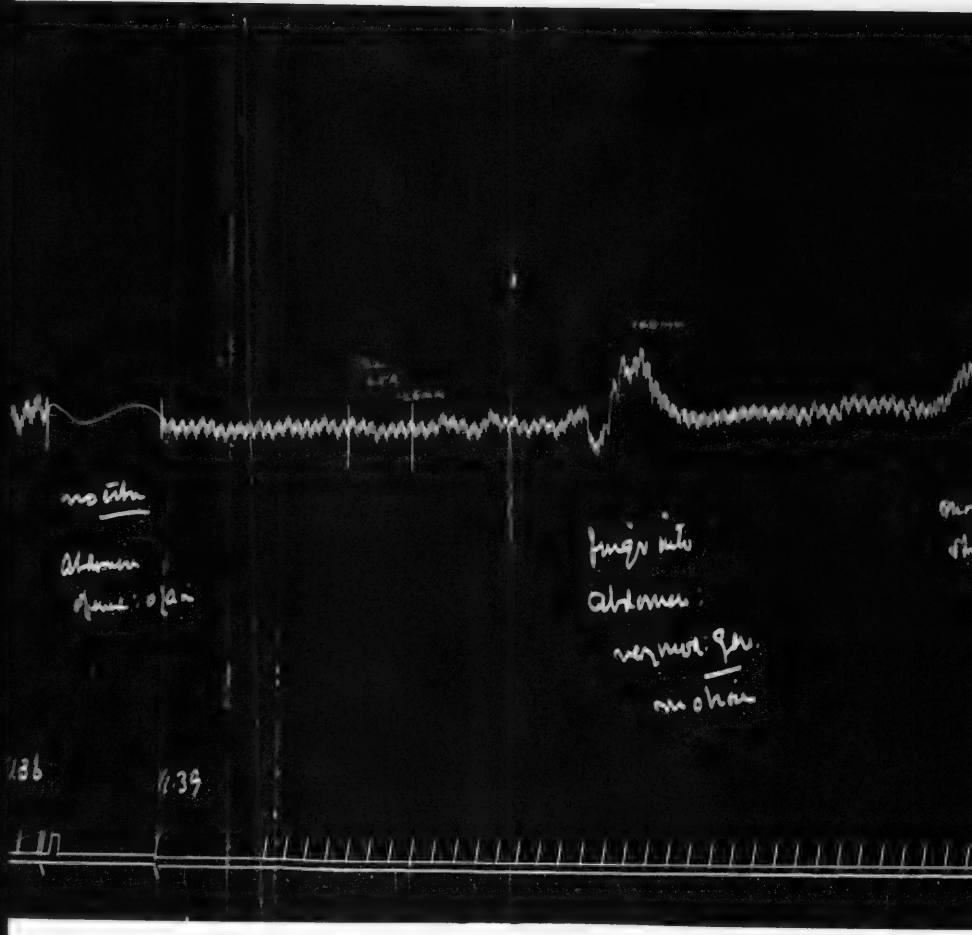
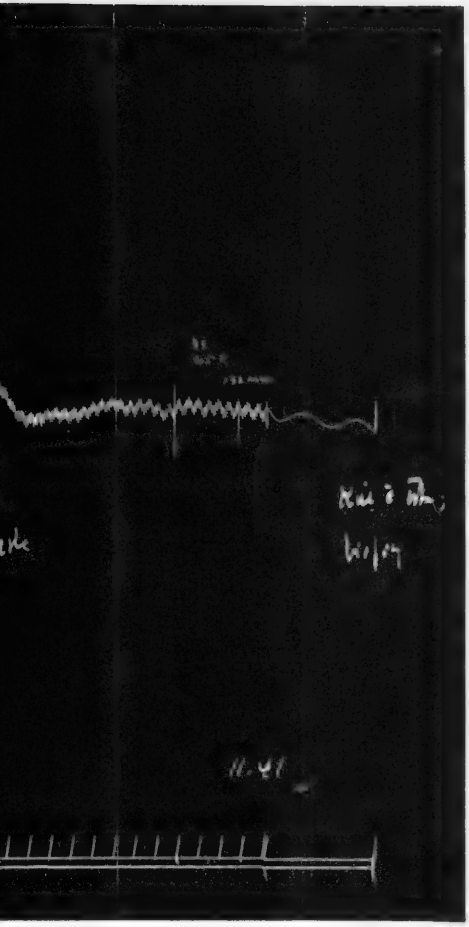

11:36
11:39

PLATE 6.



(Auer: Analgesia after exposure to war gases.)

EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

II. Survival of the Virus in Aerobic and Anaerobic Culture Media.

By PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 9, 1921.)

In the first paper of this series[1] it was shown that the typhus virus, in inducing the typical experimental disease in guinea pigs, readily invites invasion in the bodies of these animals of a number of bacteria, which complicate the typhus infection but, on the other hand, have no etiological relationship to the disease.

We thought to continue, in attacking the problem of the etiology of this disease, by means of a deductive method; that is, to put the virus to a variety of experimental tests so as to study the nature of any inciting agent which may reside therein. One of these tests will be described in this paper. The test concerns the survival of the virus in different media, with especial reference to the influence of anaerobic conditions. From the results obtained, we hoped to acquire particular facts which might serve as guides for cultivation experiments.

Method.

The following method was employed. The medium to be tested, under aerobic and anaerobic conditions, was inoculated with the virus present in the blood of a guinea pig reacting to experimental typhus fever. After keeping the mixture of medium and blood containing the virus for varying intervals at 37°C., the blood was removed and injected intraperitoneally into guinea pigs to determine the viability of the virus. Its activity was demonstrable when these guinea pigs developed typical experimental typhus fever, characterized by

[1] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 525.

(*a*) the transmissibility of the disease from animal to animal, (*b*) the presence of characteristic vascular lesions, especially in the brain, (*c*) the absence of secondary invasions with ordinary bacteria, and (*d*) the development in recovered animals of immunity to subsequent injections of typhus virus.[2] The virus was determined to be inactive when guinea pigs injected with it failed to develop either the typical experimental disease, or immunity to further injections of typhus virus.

The details of the methods of procedure were as follows:

Virus.—The virus for the following experiments was obtained from the blood of guinea pigs during the height of their reaction to experimental typhus fever. The blood was secured by cardiac puncture[3] with a sterile needle and syringe washed in 50 per cent sodium citrate solution. Three strains of virus, described in another communication,[2] were employed: human and louse strains originally derived from Poland and a human strain obtained from the blood of a Czecho-Slovakian immigrant arriving at the Port of New York.

Media.—The media used in the following tests comprised: normal rabbit plasma undiluted; normal horse serum diluted one part to three parts of saline solution; veal infusion 2 per cent peptone broth of a pH of 7.4; Smith-Noguchi tissue, ascitic fluid, sealed medium, prepared in a manner described in another paper.[4] The latter was modified as follows: in one set of experiments, omission of the tissue; in another, of the petrolatum seal; and finally, human ascitic fluid, undiluted.

Production of Anaerobic Conditions.—Comparative experiments were performed with each medium under aerobic and anaerobic conditions. An anaerobic state was produced by overlaying the medium, immediately after inoculation, with melted petrolatum. It has already been demonstrated [4,5] that this seal produces an effective anaerobic condition in a few hours in the medium underneath.

Procedure.—4 cc. of guinea pig blood, corresponding to the typhus virus, were inoculated into each of two tubes containing an equal amount of the medium to be tested. The presence of the citrate defibrinated the blood and consequently the blood settled to the bottom of the tube. One tube of the inoculated medium was left unsealed, another was overlaid with melted petrolatum (2.5 cc.). Both tubes were then kept in the same incubator at 37°C. for an indicated period of time. To test the activity of the blood, 3 cc. of the supernatant medium were removed, without disturbing the sedimented blood, and the remaining 5 cc. were

[2] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

[3] Experiments were performed upon animals under deep chloroform anesthesia.

[4] Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

[5] Hall, I. C., *J. Bact.*, 1921, vi, 1.

injected intraperitoneally into a guinea pig. The blood in the aerobic and in the anaerobic medium was injected at the same time, so as to avoid an experimental error. To avoid further error, the experiments were repeated. For similar reasons, guinea pigs which failed to show the typical reaction after injection were observed for a period of about 1 month before they were submitted to an immunity test.

RESULTS.

Virus without Addition of Medium.—As a control to aid in the interpretation of the experiments, 4 cc. of active blood, obtained from guinea pigs during the height of reaction to experimental typhus fever, were kept in a test-tube at 37°C. without the addition of any medium. The blood was kept in the incubator for 24, 48, and 72 hours. At the end of these periods, it was injected intraperitoneally into guinea pigs. Only the blood which was exposed to 37°C. for 24 hours induced typical experimental typhus fever; exposure beyond 24 hours failed to set up either the disease, or immunity to subsequent injections of the virus.

Virus Added to Broth.—Under aerobic conditions, the virus in the blood was active after remaining in this medium for periods ranging up to 5 days. After the 5th day, the virus failed to induce either the experimental disease or immunity.

Under anaerobic conditions, the virus was active after remaining in this medium for 24 and 48 hours, but failed to induce either the disease or immunity after periods longer than 48 hours.

Virus Added to Normal Rabbit Plasma.—Under aerobic conditions, the virus in the blood survived in this medium for 5 days, but failed to induce the disease or immunity in guinea pigs after longer periods.

Under anaerobic conditions, the virus survived for 24 hours, but not thereafter.

Virus Added to Normal Horse Serum.—Under aerobic conditions, the virus in the blood survived in this medium for 5 days but not thereafter.

Under anaerobic conditions, the virus survived for 24 hours, but not for longer periods.

Virus Added to Human Ascitic Fluid Media.—Under aerobic conditions, in human ascitic fluid media with or without the addition of a fragment of fresh rabbit kidney tissue, the typhus virus survived

for periods of 48 and 72 hours at 37°C., but lost its activity after longer periods of exposure.

Under anaerobic conditions, in human ascitic fluid medium without rabbit kidney tissue, the virus remained viable for a period of 48 hours, but not after a longer period. In human ascitic fluid medium, to which was added a fragment of sterile rabbit kidney tissue, and which was then sealed with petrolatum—identical with the anaerobic Smith-Noguchi medium—the virus remained active for only 24 hours. In this medium after 2, 3, 4, and 5 days, the virus failed to induce

TABLE I.

Effect of Different Media, under Aerobic and Anaerobic Conditions, on Typhus Virus.

Medium.	Condition of oxygenation.	Period of survival of virus.
		days
Broth.	Aerobic.	5
	Anaerobic.	2
Normal rabbit plasma.	Aerobic.	5
	Anaerobic.	1
Diluted normal horse serum.	Aerobic.	5
	Anaerobic.	1
Human ascitic fluid (with or without rabbit kidney).	Aerobic.	3
	Anaerobic.	2
Smith-Noguchi medium.	"	1

either the disease or immunity to further injections of typhus virus. The addition of dextrose broth to this anaerobic medium did not prolong the period of viability of the virus.

Table I summarizes these results.

These experiments show that the typhus virus, as it is found in the blood of guinea pigs reacting to the experimental disease, if kept at 37°C. in different media from which oxygen is excluded by a petrolatum seal, tends to die off rapidly, in 24 to 48 hours. On the other hand, the period of survival is prolonged to 5 days (in only one case to 3 days) when the same media have free access to atmospheric oxygen.

DISCUSSION AND SUMMARY.

The results of the foregoing experiments show that the typhus virus, found in the blood of guinea pigs during the height of typical experimental typhus fever, does not survive at 37°C. in anaerobic media for as long a period as in the same media under aerobic conditions. In media from which oxygen is excluded, the viability period is 24 to 48 hours; in the same media having no barrier to atmospheric oxygen, the period is usually 5 days, in one instance, 3 days.

The dead virus fails to induce not only the typical experimental disease but also an immunity to further injections of typhus virus.

That the death of the virus is due to exclusion of oxygen from the medium, rather than to a change in the hydrogen ion concentration therein is inferred from the fact that media with varying hydrogen ion concentrations, such as broth (pH 7.4), horse serum (pH 7.8), and human ascitic fluid (pH 8.0) have the same comparative effect on the virus, when under aerobic and anaerobic conditions. That is, in all, the anaerobic state causes a shortening of the viability period of the typhus virus.

In the Smith-Noguchi tissue, ascitic fluid, sealed medium in which bacteria resembling Plotz' bacilli grow luxuriantly and remain viable for several weeks, the typhus virus does not increase in virulence, and even dies after 24 hours. This evidence supports the conclusion previously presented[1] that the *Bacillus typhi exanthematici* of Plotz is not identical with the active agent of typhus virus.

CONCLUSION.

In a variety of media kept at 37°C., and from which oxygen is excluded by a petrolatum seal, the typhus virus tends to die rapidly. In the corresponding media under aerobic conditions, the life of the virus is appreciably longer.

EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

III. Filtration Experiments.

By PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 4, 1921.)

In a previous paper[1] we presented a plan of studying the problem of the inciting agent of typhus fever by the deductive method; that is, by applying to the typhus virus a variety of known tests in order to determine the nature of the particular incitant which may reside therein. From the experiments performed, it was concluded that the virus, in inducing experimental typhus fever in guinea pigs, invites invasion of the body of the animals by a variety of bacteria, which complicate the infection but have no etiological relationship to typhus,[2] and that the virus itself survives for a longer period in aerobic than in anaerobic culture media.[1]

In this article, the deductive method is continued and additional experiments on the typhus virus are presented relating to the filterability of the virus as it exists in the organs of guinea pigs during the height of the reaction to inoculation.

HISTORICAL.

Heretofore, most of the experiments on the filterability of typhus virus have been made with the blood taken from guinea pigs or monkeys at the height of the experimental typhus infection. Goldberger and Anderson,[3] after reviewing all previous experiments, state that there have been eight attempts, by different workers, to pass the virus of typhus through a Berkefeld filter. Of these, six were negative; in one of the other two (Wilder, 1911[4]), the monkey that had been

[1] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 115.

[2] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 525.

[3] Goldberger, J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H., No. 86*, 1912, 62.

[4] Wilder, R. M., *J. Infect. Dis.*, 1911, ix, 9.

inoculated with the filtrate, without giving any evidence of infection, was later found to be resistant to an immunity test with active blood; in the other (Nicolle, Conor, and Conseil, 1910[5]), one of two monkeys inoculated with filtrate is described as having presented a doubtful reaction, and later was found to be resistant to an inoculation with active blood.[6] Goldberger and Anderson then described their own experiments, which convinced them that the virus, as it exists in the blood, is not filterable.

Later, in 1914, Nicolle, Blanc, and Conseil[7] inoculated into monkeys filtrates prepared from lice harboring the virus, and while no typical reaction resulted, it was found that on reinjection with active blood only a delayed and mild reaction occurred, which these workers regarded as evidence of a partial immunity.

In 1917, the writer's[8] efforts to filter the virus in the blood of experimentally infected guinea pigs during the reaction were fruitless. He concluded that the virus was not filterable.

The foregoing review shows that as yet no conclusive experimental basis has been offered to prove that in typhus blood the virus is filterable, although Nicolle and his associates hold that opinion.

In 1911, Nicolle, Conor, and Conseil[9] assumed that the virus of typhus fever is an intracellular parasite. Later observers, especially those who have linked the *Rickettsia prowazeki* with the incitant of typhus fever (da Rocha-Lima,[10] Wolbach and Todd,[11] and others) have taken the same stand.

The experiments to be described relate to two points: first, the supposed intracellular nature of the inciting agent, and second, the filterability of the virus. In order to approach more nearly ideal conditions of experiment, we employed organs derived from the infected animals instead of the blood.

Methods.

The spleen and brain of typhus-infected guinea pigs at the height of their reaction were chosen. Two strains of virus from human

[5] Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1910, cli, 685.

[6] In a later communication, we shall present evidence to show that the production of immunity in isolated instances after the injection of filtrates may possibly be dependent on other factors than a living multiplying agent.

[7] Nicolle, C., Blanc, G., and Conseil, E., *Arch. Inst. Pasteur Tunis*, 1914, ix, 84.

[8] Olitsky, P. K., *J. Infect. Dis.*, 1917, xx, 349.

[9] Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1911, cliii, 578.

[10] da Rocha-Lima, H., *Arch. Schiffs- u. Tropen-Hyg.*, 1916, xx, 17.

[11] Wolbach, S. B., and Todd, J. L., *Ann. Inst. Pasteur*, 1920, xxxiv, 153.

sources were used: one originating at Warsaw,[12] the other obtained from a Czecho-Slovakian immigrant at the Port of New York. Both strains have been described in a previous report.[13] That the spleen and brain are capable of inducing the experimental disease in guinea pigs has been shown by Nicolle and Blaizot[14] as well as by later workers. Indeed, Landsteiner and Hausmann[15] have induced experimental typhus fever in guinea pigs by inoculating as small a dose as 0.005 gm. of the infected brain tissue.

The first experiments refer to the problem of the supposed intracellular location of the virus and to an attempt to free it through cellular disintegration.

Repeated Freezing and Thawing.—An infected guinea pig, at the height of its reaction, was exsanguinated.[16] The spleen was removed under sterile conditions; one-fifth was kept for a control test and the remainder finely pulped with scissors. The pulp was placed in a test-tube containing 20 cc. of Ringer's solution, and frozen solid by means of calcium chloride in cracked ice at −26°C. The frozen mass was then thawed out to the original fluid condition and immediately refrozen. A small portion of the pulp was removed and tested for growth *in vitro* with negative results.[17] In the meanwhile, the remainder of the pulp in suspension, with the exception of 2 cc. employed in the control test, was filtered through a tested Berkefeld V or N candle and the filtrate inoculated in the manner to be described.

Freezing and Desiccating.—In this test the spleen was frozen and then desiccated to a flaky powder according to the method of Swift.[18] As in the case of the previous method, controls of tissue before desiccation were employed. The remaining four-fifths of the desiccated splenic tissue was suspended in Ringer's solution and filtered after 4 cc. had been removed for control purposes.

Crushing in a Mechanical Tissue Crusher.—The same conditions existed in this test as in the above, except that the spleen was placed in a mechanical tissue crusher and the tissue juice and very fine homogeneous semifluid pulp resulting were suspended in Ringer's solution and filtered, the 2 cc. of the original unfiltered material being withheld for control purposes.

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[12] We owe this strain to the kindness of Dr. S. B. Wolbach.

[13] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

[14] Nicolle, C., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1916, ix, 127.

[15] Landsteiner, K., and Hausmann, W., *Med. Klin.*, 1918, xiv, 515.

[16] All operations were performed under deep chloroform anesthesia.

[17] This test was performed by Dr. Alexis Carrel.

[18] Swift, H. F., *J. Exp. Med.*, 1921, xxxiii, 69.

Grinding with Sand.—In the same way, the cut up fragments of spleen were ground with sterile fine white sand in a mortar, with the addition, from time to time, of 5 cc. of Ringer's solution until the required amount (20 cc.) was obtained. All but 2 cc. of the thin, even, homogeneous suspension was then filtered.

Precisely similar procedures were carried out with brain tissue from infected guinea pigs.

Of the four methods employed in treating the cellular elements of the tissues, those of repeated freezing and thawing at $-26°$C., and freezing and desiccating are known to kill the cells completely.[19] Crushing or grinding tissues destroys the cells, but it is presumed that an occasional living cell may escape. However, the following experiments demonstrate that the virus in the tissues prepared by any of these methods retains an undiminished infecting power. Hence one method serves to control the others.

There were thus available for testing (*a*) typhus-infected tissues of guinea pig spleen and brain, (*b*) the same tissues, either frozen, desiccated, crushed, or ground, and suspended in Ringer's solution, and (*c*) filtrates of these suspensions which were freed from the tissue cells and detritus.

Procedure.—Each experiment comprised six animals. Guinea Pig A was injected intraperitoneally with 2 cc. of the suspension of infected tissue. This animal served as a control to test the infecting power of the virus in the tissue.

Guinea Pig B was injected intraperitoneally with 2 cc. of the suspension of the unfiltered suspensions of infected tissue, either frozen, desiccated, crushed, or ground, in the order of the tests made. This animal served as a control to test the effect on the virus of the methods used in disintegrating the tissue cells.

Guinea Pigs C and D were injected with 5 cc. of the filtrate of the particular suspension of the tissue (either frozen, desiccated, crushed, or ground) to be tested. Of these animals, Guinea Pig C was allowed to run its course so as to detect any delayed reaction and, in certain instances, was reinjected, after a suitable interval, with active typhus virus in order to test its immunity. Guinea Pig D was killed from 4 to 7 days after injection,[20] and 3 cc. of its defibrinated blood were inoculated into each of two normal animals, E and F. In this way the possibility of transmission of a living, multiplying agent might be detected and the pathological changes in the body of Guinea Pig D studied.

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[19] For the literature on this subject, see Wells, H. G., Chemical pathology, Philadelphia, 4th edition, 1920, 373.

[20] During this period the blood of guinea pigs experimentally infected contains active virus.[2]

RESULTS.

In all, fourteen experiments, as detailed above, were made with infected spleen and brain derived from guinea pigs at the height of the experimental infection: Three were made with the frozen infected tissue; two with the desiccated tissue; three with the crushed tissue, and six with the ground tissue. The results of the different experiments were practically identical.

Guinea pigs of Series A, injected with the infected tissue which was not disintegrated, showed, after an incubation of from 5 to 10 days, typical experimental typhus fever. On transmission by means of the blood obtained during the height of the reaction to normal animals, the latter, in turn, exhibited the typical fever. This was characterized by the specific histopathological picture, immunity to subsequent injections of active virus, freedom from concurrent or secondary infections, and transmissibility of the fever to normal animals—thus agreeing with the definition of the experimental disease described elsewhere.[13] Hence the materials employed for the filtration tests were proved to have specific infecting power.

Guinea pigs of Series B, injected with the infected tissue which had been either frozen, desiccated, crushed, or ground, exhibited the typical experimental typhus fever paralleling that shown by the animals of Series A.[21] Therefore, the tissue cells killed by repeated freezing and thawing, or by freezing and desiccating, or crushed into detritus by mechanical means, or ground into a homogeneous fine pulp, have had no effect on the infecting power of the typhus virus.

Guinea pigs of Series C and D, inoculated with the filtrates of suspensions of the frozen, desiccated, crushed, or ground infected tissue, failed to show the typical experimental disease. In five experiments, the animals of Series C were reinjected with active typhus virus; all failed to show any immunity. In ten experiments, the blood of guinea pigs of Series D, obtained 4 to 7 days after injection with the filtrates, was inoculated into normal animals (Series E and F). In none of these was there induced either typical experimental typhus

[21] The unfiltered desiccated tissues were injected 6 days after drying; in the instances in which the tissues were disintegrated by the other methods, the injections were made immediately after disintegration.

fever or immunity. Hence the filtrates failed both to cause the typical disease and to produce immunity.

To summarize the results of these experiments, we find that fourteen attempts to filter the active typhus virus present in the brain or spleen of guinea pigs at the height of reaction have ended in failure.

SUMMARY AND CONCLUSIONS.

We have presented experiments to show that the typhus virus in the tissues of the guinea pig during the height of reaction to the experimental disease does not lose its infecting power when the cells of the brain or of the spleen are disintegrated by repeated freezing and thawing, or by freezing and desiccating, or by crushing by mechanical means, or by grinding into a homogeneous pulp with sand. The virus after such treatment is as actively infective as in the same tissue not subjected to the disintegrating influences. The possibility exists, therefore, of an extracellular condition of the typhus virus.

Fourteen attempts to filter through Berkefeld V and N candles the virus contained in the disintegrated tissue have all resulted in failure.

DO SPECIES LACKING A GALL BLADDER POSSESS ITS FUNCTIONAL EQUIVALENT?

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It is a singular fact that certain closely related species of animals have, some of them, a gall bladder, while others do not. What can be the essential significance of the organ and the reason for this difference?

Embryologically, the vesica fellea arises secondarily as a cul-de-sac from the same anlage that forms the liver and bile ducts, and it is considered by many as an almost purposeless diverticulum. The erroneous nature of this view is sufficiently shown by the several functions now demonstrated for the organ. The matter has been discussed in a previous paper from this laboratory.[1] The fact that the gall bladder is a highly specialized organ renders all the more noteworthy its irregular distribution in both high and low forms of life. Thus among the higher animals it is present in the cow and sheep, while it is absent in the horse, present in the goat, and absent in the closely related deer—to be found in the hog and wild boar but not in the peccary of South America. Among birds, the hawk and owl possess it, while doves do not; and among the rodents the mouse is found with the organ, the rat without. One species of gopher,[2] the pocket gopher (*Geomys bursarius*), is without a gall bladder, while another (*Spermophilus tredecimlineatus*) the striped gopher, possesses it. Woods Hutchinson[3] is authority for the statement that in the giraffe it is at times present and again not.

[1] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

[2] Mann, F. C., *J. Lab. and Clin. Med.*, 1919–20, v, 107.

[3] Hutchinson, Woods, *Med. Rec.*, 1903, lxiii, 770.

The disposition of the bile ducts or the position of the pancreas throws no light on the riddle of the gall bladder. If we consider only animals without the organ, the horse has separate pancreatic and common bile ducts emptying into the duodenum through a common opening. The deer, rat, and pocket gopher (*Geomys bursarius*) show the pancreatic duct opening directly into the common bile duct well above the ampulla, this it also does in both rats and white mice, the former being without a gall bladder, the latter with it. No inference can be drawn from the high opening of the pancreatic duct, for this is as inconstant[4] as the presence of the gall bladder itself.

The vesica fellea has, as already indicated, functions sharply different from those of the ducts. Its rôle as a reservoir may on occasion, as after cholecystectomy, be partially taken over by the ducts.[5] Not so, however, with its modifying influences upon the bile. The bladder acts to concentrate the secretion greatly and to thicken it with mucus, whereas the ducts by contrast tend to dilute it, though to a negligible degree, with a thin product of their own.[6] Is it possible that in animals lacking the gall bladder, the concentrating function is lodged somewhere in the duct wall? This possibility can readily be tested out by ligation experiments on rats and mice.

If the gall bladder has the same activities in the mouse as in the dog, cat, and monkey,[1] then the bile submitted to its influence after ligation of the common duct should become concentrated by the action of the gall bladder. Granting that this really occurs,—and the findings now to be described show that it does,—will a concentration of bile also go on in the obstructed duct system of the rat, an animal devoid of a gall bladder, or will this bile be diluted as is the case with bile pent in the ducts of other animals which have been deprived of their connection with the bladder? The close relationship of the species and their similarity in habits give a special value to the test.

[4] Mann, F. C., Foster, J. P., and Brimhall, S. D., *J. Lab. and Clin. Med.*, 1919–20, v, 203.

[5] Haberer, H., and Clairmont, P., *Verhandl. deutsch. Ges. Chir.*, 1904, xxxiii, pt. 2, 81. Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 710.

[6] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 75.

Method.

The pigment content of the bile was taken as the criterion of extrahepatic changes in concentration. Its utility in this connection has already been shown.[1] The range of the normal pigment content of mouse and rat bile was first determined with the animals on their ordinary ration (bread and milk, and grain).

From rats the bile was obtained into small rubber bags connected with fine cannulas inserted in the common duct. The bags were left within the animal's peritoneal cavity for 24 hours or more. Considerable amounts of secretion, ample for pigment analyses, were readily obtained in this way. The small size of the mouse, however, made necessary a different method, which will later be described.

All operations were carried out under ether, with aseptic precautions. To produce stasis, a triple ligation of the duct was done, the ligatures being placed just below the junction of the lowest hepatic duct with the common duct and well above the pancreatic duct entrance, and the common duct was then cut between the middle and lowest ties. The animals were allowed to live thereafter for periods of 1 to 16 days on a mixed diet of barley and oats, with bread and milk. Recovery from the operative procedure was rapid and the animals ate well. They were killed at intervals, the stasis bile was collected into a pipette, and its pigment content determined. In no instance had the continuity of the duct been reestablished. Cultures of the bile and liver tissue were regularly taken.

The Quantitation of Pigment.

Different methods were used to determine the pigment content of bile according to the amount available. When more than 0.3 or 0.5 cc. of rat bile was had, a reading was carried out according to the colorimetric method of Hooper and Whipple.[7] To a measured quantity of bile is added a known quantity of acid alcohol reagent. For a standard, instead of a wedge of artificial constitution, a chloroform solution of pure bilirubin (Schuchardt) was used, which was mixed with the reagent, as was the bile, and read against it in a Duboscq colorimeter.

When the quantity of rat bile was very small, the mixture of it with the acid alcohol reagent was made in a Miescher pipette such as is used in the Fleischl-Miescher hemoglobinometer and read against the standard bilirubin solution as ordinarily, but in a micro colorimeter.

[7] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

The pigment content of normal mouse bile in the small quantities obtained was always insufficient to yield, with the reagent, a green color that could be read in the colorimeter. It was quantitated directly against a potassium bichromate scale, a method used as well with rat biles. For the purpose, glass tubes with a bore of 0.75 mm. and walls so thick (2 mm.) as to result in a magnification, were filled with bile and with the scale solutions. The tint of bile and standard matched very closely. A permanent series of tubes was made containing dilutions of potassium bichromate ranging from 6.0 to 0.05 per cent; and tests were made to determine whether the color intensity of mouse bile varied on dilution like that of a weak bichromate solution. Specimens of bile which corresponded in color with bichromate solutions of known percentage were diluted and compared again with the scale. They now agreed with a percentage bichromate solution corresponding to their dilution. Thus a bile solution corresponding in color with a 1 per cent bichromate solution, when diluted with equal parts of water, had the color strength of a 0.5 per cent bichromate solution—when with ten parts of water it corresponded with a 0.1 per cent solution, and so on. In order to standardize the scale, a number of normal rat biles of known pigment content, taken at random, were compared with the bichromate tubes. The results of this comparison have been assembled in Table I. There three subdivisions have been made according to the pigment strength of the bile employed. The bilirubin content in milligrams per 100 cc., as determined by the acid alcohol reagent, is given in Column A, and its reading in percentage of bichromate solution, as determined by comparison with the small glass tubes, is given in Column B. If the relative amount of pigment can be read as truly on the bichromate scale as by the Hooper and Whipple method, then $\frac{A}{B}$ should yield a constant, which constant incidentally is the expression in milligrams of bilirubin per 100 cc. of bile, of the colorimetric value of a 0.1 per cent bichromate solution, or for a 1 per cent solution, when multiplied by 10. It will be seen that the constant was only approximately yielded by the individual bile specimens, but the variation is much the same throughout the groups, and the average for the constant was 13.26, 13.51, and 13.57 for biles of

TABLE I.

Standardization of the Potassium Bichromate Scale.

Rat No.	A Actual amount of bilirubin per 100 cc.	B Per cent strength of bichromate solution corresponding in color to the bile.	$\frac{A}{B} \times 10$	Averages.	
	mg.				
8	8.9	0.7	12.7		
37	11.7	0.8	14.6	Pigment less than 15 mg. per 100 cc. of bile.	
38	12.5	0.9	13.77		
9	14.38	1.2	11.98	Average 13.26	
7	15.43	1.4	11.02		
6	16.8	1.4	12.0	Pigment amounts 15 to 20 mg. per 100 cc. of bile.	
14	17.85	1.2	14.88		13.45
15	19.1	1.2	15.91	Average 13.51	
10	19.23	1.4	13.75		
5	20.16	1.8	11.2		
16	20.83	1.5	13.88	Pigment amounts above 20 mg. per 100 cc. of bile.	
13	22.32	1.6	13.9		
11	22.72	1.5	15.15	Average 13.57	
12	24.51	1.8	13.6		

All bile collections were made by the bag-cannula method.

Rat biles collected by the bag-cannula method were employed. In Column A, the actual milligrams of pigment per 100 cc. of bile, as obtained by the acid alcohol method, are given. Column B gives the pigment strength of the bile in per cent of potassium bichromate solution as obtained by direct color comparison. If this latter comparison is a true one, then $\frac{A}{B}$ should yield an expression in milligrams of bilirubin per 100 cc. of bile of the colorimetric value of a 0.1 per cent bichromate solution, as determined from that particular specimen of rat bile. This multiplied by 10 should give the figure for a 1 per cent solution ($\frac{A}{B} \times 10$), and should be a constant. The readings are separated into three groups according to whether the pigment content of the bile fell below 15 mg. per 100 cc., between 15 and 20 mg., and over 20 mg. per 100 cc. It will be seen that the same approximate constant ($\frac{A}{B} \times 10$) is yielded by all three groups.

low, medium, and high pigment content respectively, the general average being 13.45. As the table shows, a bile which reads on the bichromate scale at 1 per cent will contain about 13.45 mg. per 100 cc. Thus a specimen of rat or mouse bile obtained from a duct could be placed in one of the small bore tubes, compared with the bichromate scale, and its pigment content quantitatively estimated.

The Pigment in Normal Mouse Bile.

The delicacy of the common duct in mice prohibited the use of the bag and cannula method which was successful with rats. Instead, bile was collected from animals under ether, through extremely fine long cannulas, with the aid of suction.

To obtain cannulas of sufficient flexibility, hard glass tubing was drawn out into capillary pipettes with an external diameter of about $\frac{1}{3}$ mm. and a length of 40 to 60 cm.. with the small end carefully rounded to avoid laceration of the duct. The larger end of the tube was curved and the bend in it formed a sort of basin into which the bile from the capillary collected. Mild suction was maintained at this end, when desired, through a connection with a rubber tube and bulb filled with water, which could be lowered at will.

The larger end of the cannula was rigidly attached to a mechanical stage which permitted accurate movement in two dimensions of space and its flexible shaft was passed through three fixed wire loops, so arranged as to give it a marked downward curvature. All of the loops could be raised or lowered at will, thus increasing or decreasing the curvature of the shaft and correspondingly deflecting the open end of the cannula, so that it could be made to take the exact direction of the bile duct in the individual mouse. This adjustment was essential to prevent kinking and obstruction of the duct.

Under ether, the bile duct was exposed close to its entrance into the duodenum. By the use of the mechanical stage the cannula was then introduced either through a slit in the duct or by ligating the duodenum to either side of the papilla of Vater, opening it and thrusting the cannula through the papilla into the duct. A magnifying glass placed above the field of operation was found of service. The cannula once in the duct was always advanced to a point well above the entrance of the channel and in many instances tied in place, thus blocking off all pancreatic secretion. Often it plugged the duct so tightly that this was unnecessary. Mild suction was produced on the capillary pipette and the animal kept under ether for several hours while the flow of bile went on.

By this arrangement the gall bladder was left in direct connection with the cannula and *a priori* one would suppose that its contents

formed part of the normal bile collected through this latter. However, on first opening the abdomen to insert the cannula, the gall bladder was usually noted to be empty. Even when full, little of its contents was yielded to the cannula on suction.

Collections were successfully made by this method for periods as long as $6\frac{1}{2}$ hours. Body warmth was kept up by electric lights placed near the animal, and injury was combated with moist sponges. The bile obtained was very uniform in character, exceedingly abundant, clear, light amber-yellow but, as already said, too weak in pigment, in the small quantities obtainable, to yield a notable blue-green color with the acid alcohol reagent.

Table II shows the result of readings against the bichromate scale with the estimated amounts of bilirubin per 100 cc. of bile. An average from ten cases gives a pigment content of 2.17 mg. of bilirubin per 100 cc. of bile. The quantity of bile secreted per 100 gm. of body weight in an hour was about 0.2 cc., or, per kilo of body weight per day (24 hours), 34.0 cc. A gram of liver tissue of the mouse secreted about 0.03 cc. of bile per hour.

Results of Obstruction in Mice.

The bile was studied from many mice in which obstruction had been produced by ligation of the common duct. Obstruction lasting 1 to 3 days did not result in a marked dilatation of the ducts but the gall bladder was always found full of a bile much darker and more mucinous than normal mouse bile. It had early a clear, dark amber color, and the longer the obstruction the darker the bile. After 3 days, the ducts became dilated, though the gall bladder did not greatly increase in size. Both now contained abnormally dark bile in greater and greater amount from day to day. The actual quantity was difficult to measure, since in puncturing the ducts some was almost always lost.

In accordance with expectations based on the findings in larger animals with gall bladders,[1] the stasis bile always contained much more than the normal amount of bilirubin (Table III). When stasis had existed for more than 24 hours, the gall bladder bile had sometimes a dark greenish tint. This change of some of the pigment to biliverdin caused difficulty in the comparison with the bichromate

scale. Fortunately, the pigment amount was so great as to yield, even in the small quantities of bile at hand, the acid alcohol reaction of Hooper and Whipple whereby biliverdin and bilirubin are quantitated together.

Table III shows that the bile obtained after 1 day of stasis ranged in color strength, as expressed in terms of the bichromate scale, from 1.5 per cent bichromate to 4 per cent, with an average of 2.25 per

TABLE II.

Pigment Content of Normal Mouse Biles.

Mouse No.	Mouse weight.	Liver weight.	Period of collection.	Total bile.	Quantity of bile per 100 gm. of body weight per hr.	Quantity of bile estimated per kilo of body weight per day.	Quantity of bile per gm. of liver per hr.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bichromate scale.
	gm.	*gm.*		*cc.*	*cc.*	*cc.*	*cc.*		*mg.*
1	23.0	1.15	2 hrs., 45 min.	0.1+	0.158	38.0	0.032	—	—
2	21.6	1.4	4 " 15 "	0.06 (incomplete).	0.065	15.6	0.011	0.1	1.35
3	20.0	1.4	1 hr., 25 "	0.15	0.53	12.7	0.075	0.1	1.35
4	24.5	1.9	4 hrs., 20 "	0.29	0.273	65.5	0.035	0.1	1.35
5	23.3	1.2	5 " 5 "	0.14	0.118	28.3	0.023	0.15	2.02
6	24.0	1.2	2 " 50 "	0.04	0.059	14.2	0.012	0.3	4.03
7	27.0	1.9	2 " 50 "	0.12	0.157	37.6	0.022	0.2	2.69
8	24.0	1.8	5 " 20 "	0.32	0.25	60.0	0.033	0.1	1.35
9	—	—	5 " 40 "	0.18	—	—	—	0.25	3.36
10	—	—	3 " 10 "	0.05 (incomplete).	—	—	—	0.15	2.02
Average					0.20	34.0	0.03	0.16	2.17

cent. In normal animals, by contrast, (Table II) the range was from 0.1 to 0.3, and averaged 0.16 per cent. The stasis bile, then, was fourteen times as strong in pigment as the normal. After 1 day of stasis, no perceptible conversion of bilirubin to biliverdin had occurred.

In a number of cases bile found in the gall bladder was compared with that collected from the ducts of the same animal. The pigment strength of the bladder and duct biles were compared with the aid of the bichromate scale (Table IV).

TABLE III.

Pigment Content of Mouse Biles from Obstructed Ducts.

Mouse No.	Mouse weight.	Liver weight.	Period of obstruction.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bichromate scale.	Actual amount of bilirubin per 100 cc. of bile (acid alcohol method).
	gm.	*gm.*	*days*		*mg.*	*mg.*
11	27.8	2.25	1	2.0	26.89	—
12	23.0	1.7	1	4.0	53.78	52.08
13	19.5	1.2	1	2.0	26.89	—
14	24.2	2.2	1	1.5	20.17	—
15	21.5	1.35	1	2.2	29.58	26.04
16	27.75	1.55	1	—	—	82.6
17	—	—	1	—	—	47.7
18	25.4	2.1	1	2.0	26.89	27.77
19	25.5	1.85	2	—	—	—
20	23.0	1.2	2	—	—	52.1
21	25.0	1.9	2	—	—	38.1
22	—	—	13	—	—	32.0
23	—	—	14	—	—	35.0
Average.....				2.25 (after 1 day of obstruction only).		

Average pigment content of bile after 1 day of obstruction is 30.7 mg. per 100 cc.

TABLE IV.

Comparison of the Pigment Strength of the Bladder and Duct Biles from Mice at the Close of the Bile Collection.

Mouse No.	Results.
4	Gall bladder bile showed 7 times the concentration of duct bile after a 4 hrs. collection.
5	" " " " 4 " " " " " " " " 5 " "
6	" " " " 3 " " " " " " " " 3 " "
7	" " " " 5 " " " " " " " " 3 " "
8	" " " " 5 " " " " " " " " $5\frac{1}{3}$ " "
9	" " " " 4 " " " " " " " " $5\frac{3}{4}$ " "
Average.	" " " " 4.66 " " " " " " " " 4.3 " "

Normal and Stasis Biles of the Rat.

The rubber bags used to obtain normal bile from rats were made out of finger cots and attached to glass cannulas of capillary diameter, with the ends well rounded and the shaft curved to avoid torsion on the duct. The cannula was fastened in the duct with ligatures above the entrance of the pancreatic duct, and in some instances the pancreatic duct as well was tied. Such fat necrosis as sometimes followed this latter procedure seemed not to affect the animal materially in the short period of bile collection. The rats ate well after the operation. At the end of 18 to 24 hours they were killed with chloroform.

To produce obstruction, ligature and severance of the common duct was employed. Bile leaks into the peritoneal cavity seldom were found and animals in which they occurred were discarded. Tissue icterus always appeared in 24 hours and bile pigment was plentiful in the urine. The twenty operated animals were killed at intervals ranging from 1 to 16 days after obstruction.

At autopsy the degree of dilation of the obstructed bile ducts was noted and the approximate amount of bile present within them. Very occasionally positive cultures were obtained from bile or liver tissue of the rats into which bags had been inserted, but, probably owing to the short period of collection, such biles seemed otherwise normal. The not infrequent infected cases among the animals with obstruction were ruled out. The collecting bags gave an abundant yield of bile—never less than 1.5 cc. and as much as 6.5 cc. per 100 gm. of body weight in 24 hours.

From Table V it will be seen that the pigment content of normal rat bile as determined in sixteen animals was 17.05 mg. of bilirubin per 100 cc., with a variation of 8.9 to 22.3 mg. It is of interest to note that the bulk of bile secreted per gram of liver tissue per hour in rats and mice is almost identical, but the proportion of liver weight to body weight in rats, 1 to 21.7, is considerably smaller than that of mice, 1 to 14.6. The quantity of bile secreted per 100 gm. of body weight was, per hour, 0.151 cc., or per kilo of body weight per day (24 hours) 36.3 cc.; and of bile per gram of liver per hour 0.034 cc., or per day 0.82 cc.

The normal rat bile was always a clear amber fluid as viewed in a test-tube, quite transparent, and a brilliant light yellow when placed in one of the reading tubes for comparison with the bichromatic scale. It showed remarkably little variation in pigment concentration, but had as a rule relatively less color when the amount of bile

TABLE V.

Normal and Stasis Biles of the Rat.

Pigment content of normal biles.											Pigment content of biles from obstructed ducts.						
Rat No.	Rat weight.	Liver weight.	Period of collection.	Total bile.	Quantity of bile per 100 gm. of body weight per hr.	Quantity of bile estimated per kilo of body weight per day.	Quantity of bile per gm. of liver per hr.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bichromate scale.	Actual amount of bilirubin per 100 cc. of bile (acid alcohol method).	Rat No.	Rat weight.	Liver weight.	Period of obstruction.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimat d from reading of bichromate scale.	Actual amount of bilirubin per 100 cc. of bile (acid alcohol method).
	gm.	*gm.*	*hrs.*	*cc.*	*cc.*	*cc.*	*cc.*		*mg.*	*mg.*		*gm.*	*gm.*	*days*		*mg.*	*mg.*
1	166	8.7	25	3.6	0.087	20.8	0.017	—	—	13.87	17	109.0	6.0	1	1.3	17.48	—
2	158	7.5	25	5.7	0.144	34.5	0.030	—	—	12.33	18	99.0	5.0	1	1.2	16.14	—
3	122	7.0	22½	7.1	0.258	62.0	0.045	—	—	13.55	19	88.7	4.4	1	1.6	21.51	—
4	160	9.5	22	6.2	0.176	42.6	0.030	—	—	13.15	20	92.5	4.2	1	1.4	18.82	—
5	—	—	19	2.4	—	—	—	1.8	24.20	20.16	21	76.5	3.7	2	1.2	16.14	—
6	92	5.8	—	—	—	—	—	1.4	18.82	16.8	22	77.5	5.5	2	1.3	17.8	—
7	178	7.2	18	2.5	0.078	18.7	0.019	1.4	18.82	15.43	23	92.2	5.5	3	1.2	16.14	—
8	163	7.7	22	7.8	0.218	52.2	0.046	0.7	9.41	8.93	24	80.8	5.1	3	1.3	17.8	—
9	158	6.1	22	—	—	—	—	1.2	16.13	11.98	25	—	—	4	1.8	24.2	—
10	163	6.5	18	4.8	0.164	39.4	0.041	1.4	18.82	19.23	26	93.2	4.7	5	1.8	24.2	25.75
11	170	6.6	18	2.9	0.095	22.8	0.024	1.5	20.17	22.72	27	—	—	5	—	—	14.0
12	158	5.8	18	1.8	0.063	15.1	0.017	1.8	24.20	24.51	28	—	—	5	—	—	15.4
13	153	6.5	18	3.1	0.113	27.1	0.027	1.6	21.51	22.32	29	—	—	5	—	—	10.68
14	107	4.2	18	5.2	0.271	64.9	0.068	1.2	16.14	17.85	30	93.9	5.8	7	2.0	26.89	26.31
15	167	6.9	18	4.1	0.136	32.8	0.033	1.2	16.14	19.1	31	170.0	8.1	10	1.5	20.17	17.86
16	158	6.2	18	4.6	0.162	38.9	0.041	1.5	20.17	20.83	32	—	—	10	1.5	20.17	14.2
											33	166.2	8.0	12	0.8	10.76	13.88
											34	150.7	7.8	12	0.8	10.76	12.5
											35	—	—	12	—	—	18.4
											36	—	—	16	—	—	18.45
Average.	151.3	6.95			0.151	36.3	0.034		18.71	17.05						18.6	17.02

Proportion of liver weight to body weight in the rat is 1 to 21.7. For this average only the data of normal bile collection rats were used.

secreted was great. The stasis bile was closely similar. No change from yellow to green took place such as occurs in the dog and cat, and occasionally in mouse bile after long obstruction. Though the dilatation of the ducts became greater each day, the pigment strength of their contents was never more than that of normal bile, and as a matter of fact as time passed the concentration of pigment approximated the lowest normal figures, probably as a result of dilution with fluid elaborated by the duct wall, as in the case of dogs and cats.[6] In contrast to these results, stasis bile of the mouse always underwent a marked concentration, as will be recalled.

To find out whether there might be an early concentration of bile in the duct, followed by a later dilution as indicated, the sequence of changes in the bile after obstruction was carefully followed by means of animals killed on successive days. After the 5th day of obstruction, sufficient bile could be collected in the duct for a quantitative reading with acid alcohol, but in all cases the bichromate scale was used as with the animals killed earlier. The results in this sequence of animals are distributed amidst the earlier ones in Table V, with which they agree completely. The final average of 17.02 mg. of bilirubin per 100 cc. of bile in obstructed instances, as determined by the alcohol reagent, is remarkably similar to the figure obtained for normal bile—17.05 mg. The bichromate reading of 18.6 mg. per 100 cc. of bile also compares with the result found in non-obstructed cases of 18.7 per 100 cc. It is thus seen that rat bile undergoes no concentration after leaving the liver, even during long stasis. The secretion is about eight times as strong in pigment as mouse bile obtained from the ducts.

From these findings it is clear that there resides in the rat ducts no ability to concentrate bile such as resides in the gall bladder of the mouse. The dilatation of the rat ducts was, relatively speaking, enormous, and progressed with such regularity that one could almost state from it the number of days of obstruction. In several instances the common duct reached a diameter of 15 mm. in its broadest portion and it contained in one case more than 2.8 cc. of bile which, though still of a clear pale amber and a normal pigment content, contained more mucus than normal bile. In some animals the intrahepatic dilatation of the ducts was so great that the parenchyma had become

merely a sort of covering upon them. The liver tissue showed marked cirrhosis and was clay-yellow. Histologically the condition seen was similar to that found in dogs after long obstruction—a perilobular cirrhosis.

Influence of Diet.

The amount of bile secreted by the rat (36.3 cc. per kilo of body weight per day) and by the mouse (48.3 cc.) is not nearly as great as is put out by the rabbit and guinea pig, but is more than the output of the dog and cat. Thus, according to Quincke and Hoppe-Seyler[8] the rabbit secretes 136.8 gm. of bile per kilo of body weight a day and the guinea pig 175.8 gm., while the dog yields but 20.0 gm. and the cat 14.5 gm. per kilo of body weight. The large bile output of the Herbivora has usually been ascribed to their diet; and our animals were on a vegetable ration. To determine the importance of this factor, several rats were placed on a meat diet for a period of 17 days, and bag collections were then made as in normal grain-fed animals. But the bile obtained was not unusual either in amount or character.

SUMMARY.

In a previous paper the point has been brought out that the influence of the gall bladder upon the bile differs entirely from that of the ducts, the one organ acting to concentrate the secretion markedly and the other to dilute it slightly. The question arises, in species lacking a gall bladder, whether the concentrating function of this organ will be found lodged in the ducts. To test the point, observations have been made upon the mouse and rat, since these animals though so nearly related have, the mouse, a gall bladder and the rat, none.

The normal bile was first studied. Both animals were found to secrete larger quantities than do cats and dogs, but less than the guinea pig and rabbit. Methods were worked out for the quantitation of the pigment which was used as the index to changes in concentration.

[8] Quincke, H., and Hoppe-Seyler, G., in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1899, xviii, 59.

Bladder bile of the mouse was regularly found to be more concentrated than that collected from the common duct of the same animal. The bile collecting during stasis regularly showed a great increase in pigment content, such as in other species is brought about by the action of the gall bladder. In the rat, on the other hand, stasis bile never became more concentrated in pigment than the normal.

The gall bladder, then, is not only absent from the rat in form, but in one at least of its important functions. That its other obvious function—that of a reservoir—cannot be assumed in the rat by the ducts would seem to be indicated, not only by the small size of these channels, but by the recent observation of Mann[2] that the tonus of the sphincter of Oddi is almost negligible in the rat, in contradistinction to animals which possess a gall bladder.

It is an interesting fact that the bile of the rat, which as has been said, undergoes no condensation of bulk after leaving the liver, contains on the average eight times as much pigment as does the liver bile of the mouse which is submitted to concentration. Whether it is correspondingly strong in substances useful for digestion, and therefore *ab initio* requires no concentration, is a matter upon which little can be said at present. However, in this connection the fact that the bulk of bile secreted per gram of liver weight is identical in both animals may be significant. Although this output is the same, the mouse liver when compared with the body weight (1 to 14.6) is relatively larger than that of the rat (1 to 21.7), so that the mouse secretes somewhat more bile per 100 gm. of body weight. This bile as it comes from the liver is but one-eighth as strong at least in pigment as rat bile, but the concentrating activity of the gall bladder is so great that the products yielded to the intestine may become not dissimilar.

SOURCES OF THE ANTIBODIES DEVELOPING AFTER REPEATED TRANSFUSION.

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The recent wide utilization of transfusion as a therapeutic measure has brought to light many facts of theoretical as well as practical significance. Perhaps most interesting from both points of view is the gradual decrease in beneficial effect, and the appearance sometimes of positive injury from the frequently repeated injection of alien blood. Were it not for this complication one might reasonably expect to maintain cases of pernicious anemia in good blood condition for an indefinite period of time. Needless to say, the sources of the failure have been the subject of much discussion and of some research. One fact of great weight has been clearly shown. Blood derived from a donor originally compatible, as proven both by *in vitro* tests and by the clinical result, may not only cease to be useful when too frequently injected into the same individual but may give rise to serious reactions. A change has occurred, not in the donor but in the recipient, such that the alien blood is no longer tolerated in circulation.

Boycott and Douglas[1] have noted that blood is destroyed more rapidly after repeated transfusions in normal animals than it is at first, as attested by an increase in the rate at which plethora disappears. One of us, with Oliver,[2] has utilized the phenomenon to induce in rabbits a hemosiderosis closely resembling that of hemochromatosis in man. No doubt a part of the pigmentation observed at autopsy in pernicious anemia patients who have been repeatedly transfused is due to a like destruction of alien blood. But how is this blood destroyed? By circulating antibodies or within special organs?

[1] Boycott, A. E., and Douglas, C. G., *J. Path. and Bact.*, 1909, xiii, 414.

[2] Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

Recent observations indicate that circulating antibodies are to a considerable degree involved.[3] It has been found that repeated transfusions of compatible blood in rabbits are followed often by the appearance in the recipients' plasma of hemagglutinins so strong that the red cells come together into a firm mass practically as soon as the blood has been shed, while, furthermore, a fulminant destruction of corpuscles may take place *in vivo* with result in anemia. Robertson[4] has presented evidence that the elements destroyed are the alien cells which, little by little, under the circumstances of plethora and diminished bone marrow activity consequent thereon have taken the place of cells proper to the host.

The hemagglutinins just mentioned are in the immediate sense autoantibodies. They clump practically all of the circulating erythrocytes, are especially effective at low temperatures, and persist in high titer for months after the transfusions have been discontinued and after recovery from the severe intercurrent anemia which may develop soon after their appearance. Is it possible that an organism engaged in eliminating unusually large amounts of blood will elaborate antibodies directed against its own cells? This is a point of major interest in any study of the source of the hemagglutinins, and one not without a practical bearing. In human beings true autohemagglutinins have repeatedly been observed[5] in association with anemia of obscure origin; while autohemolysins are known to bear an important relation to paroxysmal hemoglobinuria.

Transfusion Method.

Three to six compatible donors were selected for each recipient by the examination of mixtures of the citrated bloods.[6] The recipients received, 6 days in every 7, 10 cc. of blood taken by cardiac aspiration into 1 to 2 cc. of 0.9 per cent salt solution containing 1 per cent of sodium citrate. The donors were employed in rotation. The small amount of citrate mentioned was sufficient to prevent clotting during the short period required to introduce the blood into the recipient's ear vein. About half of the transfused animals failed to develop autoagglutinins at any time, even when the injections were continued for many weeks. In the

[3] Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1918, xxvii, 509.
[4] Robertson, O. H., *J. Exp. Med.*, 1917, xxvi, 221.
[5] Clough, M. C., and Richter, I. M., *Bull. Johns Hopkins Hosp.*, 1918, xxix, 86.
[6] Rous, P., and Turner, J. R., *J. Am. Med. Assn.*, 1915, lxiv, 1980.

other individuals they appeared early, as a rule after only five to ten transfusions; and when three or four more had been given, that is to say, after some 10 days to 3 weeks in all, they were well marked. The shed blood of the rabbits, when examined at room temperature, now showed prior to clotting a massive clumping of the red cells due, as our previous work has shown, to the presence in the plasma of true hemagglutinins.[3]

The Mixed Content of the Blood.

The transfused rabbits became, as it were, mixing vessels for several alien corpuscles and plasmas, perhaps indeed concentrators of immune principles through the elimination of the fluid wherewith the latter were introduced. The clumping of the cells of the shed blood might conceivably have been the result of:

1. The injected sodium citrate—a possibility ruled out by negative findings in control animals given citrate alone.

2. Antibodies introduced with the donor's plasma and (*a*) active against the recipient's cells. These might either have developed during the period of experiment, or have been so weak as to escape detection *in vitro*. No tests had been made to rule out (*b*) interagglutination among the donor bloods.

3. Antibodies elicited in the recipients and directed against (*a*) the strange corpuscles and (*b*) against the animal's own corpuscles.

4. Changes in the circulating cells resulting in a greater agglutinability.

5. Antibodies within the injected corpuscles, liberated by their destruction.

More remote possibilities could be invoked, as for example, a nonspecific agglutination brought about through physical changes in the plasmas pooled *in vivo*, but, as will be shown, those above listed suffice to account for the findings.

Tests with Preserved Cells.

In an initial series of experiments for the analysis of the conditions, blood specimens were taken aseptically from the individuals of several donor-recipient groups prior to any transfusions, and set aside in a citrate-glucose mixture in which rabbit corpuscles remain viable

for several weeks.[7] Later when the transfusions had elicited the clumping phenomenon, these preserved corpuscles were several times washed, made to a 5 per cent suspension with salt solution, and used in agglutination tests paralleling others carried out at the same time with freshly taken cells of the same animals.

For the purpose, equal parts of cell suspension and serum were mixed, allowed to stand for 15 to 30 minutes at room temperature, and examined microscopically. Room temperature is far more favorable to hemagglutination than blood heat. Clumping of the cells is ordinarily completed in about 10 minutes.

The tests yielded clear-cut results. Prior to transfusion no agglutination reaction was ever observed between the bloods of the prospective donors and recipients, when examined either according to the method just described or by that with whole citrated bloods previously referred to. Weak interagglutinations were sometimes noted between the bloods of certain donor groups but they were entirely absent from others. The fresh and preserved cells of the same individual were regularly found to behave almost identically on test *in vitro*. No evidence was obtained for possibility No. 4 of those above listed, namely an increase in agglutinability of cells circulating in an alien organism; but several other causes for the clumping were readily demonstrated. Most important, and most frequent, were isoagglutinins, often of high titer, developing in the recipient and effective against the cells of one or more donors (possibility 3, *a*). Such antibodies commonly appeared, and were readily demonstrable in serum separated from the clot at room temperature. Their association with the most marked clumping noted makes it highly probable that the sudden anemia supervening on plethora which occurs only in association with such marked clumping is due to isoantibodies.

Agglutinins interactive between the donor bloods (possibility 2, *b*) sometimes developed, but they were never strong. Occasionally agglutinins for the recipients' cells appeared in the donors' plasma during the period of transfusion (possibility 2, *a*). These were always so weak as to be negligible *in vivo*.

There remained a series of instances in which, with all such antibodies ruled out, there were yet hemagglutinins in the recipient that

[7] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

led to a marked clumping not only of the mixture of cells present after transfusion but of the recipient's cells taken prior to transfusion and kept *in vitro*. Such antibodies, like true autoagglutinins,[8] were readily bound to, and completely freed from, the cells by cooling and warming respectively. Tests on their origin were now begun; and to rule out wholly the possibility that undetected antibodies had been injected with the donors' bloods, the transfusions were carried out with washed cells.

Transfusions with Washed Cells.

Five donor-recipient groups were chosen in the usual way and the donors bled in rotation. 10 cc. of whole blood was taken each time into about 20 cc. of a sterile isotonic solution of sodium citrate (3.8 per cent) in water and allowed to stand in the ice box for 2 days, during which period practically complete sedimentation occurred. The supernatant fluid was now pipetted off and the cells were suspended in about 20 cc. of Ringer's solution, to which had been added 0.125 per cent of gelatin and 0.2 per cent of sodium citrate. The gelatin prevented mechanical injury of the cells during the manipulations, and the citrate did away with the slight tendency to clotting. After a further 2 days of sedimentation in the cold the corpuscles were again suspended, but this time in gelatin-Ringer's solution lacking citrate, and immediately thrown down with the centrifuge; made to a total bulk of 10 cc. with an ordinary Ringer's mixture containing only 0.76 per cent NaCl; and injected. During all the handling no hemolysis occurred except occasionally to a negligible degree in the case of a donor with unusually frail cells.

Prior to the first transfusion, blood specimens from the prospective recipients were taken for preservation as already described.

In three of five rabbits repeatedly injected with the washed cells a well defined clumping in the shed blood was soon noted. In the two better marked cases the serum of the recipient, separated from the cells at 37°C., caused at room temperature outspoken agglutination of the preserved cells of the same individual. The principle which caused the red cells to come together remained fixed upon them in the cold, and the cell masses could be repeatedly washed in salt solution without its liberation. But when the fluid was warmed to body heat the agglutinins passed into it and the cell mass separated into its components. By such means the antibody proved readily obtainable in salt solution, as is the case with autoagglutinins.[8]

[8] Landsteiner, K., *Münch. med. Woch.*, 1903, l, 1812.

In considering these results one asks first whether they might not have been duplicated with normal blood. For it will be recalled that Landsteiner[8] was able to demonstrate autoagglutinins in the normal blood of several animal species, among them the rabbit. But, the blood of our recipients showed no clumping prior to the transfusions. Furthermore, Landsteiner's report shows that his agglutinins were vastly weaker than ours, being demonstrable at room temperature only when a large amount of serum was allowed to act on a few corpuscles. Nevertheless, we made attempts to demonstrate autoagglutinins in five normal rabbits by the same measures that had been employed with the transfused ones, but obtained consistently negative results.

Autofixation of Isoantibodies.

A remote possibility which suggested itself to account for the autoagglutination just described was the fixation on the donor cells and persistence throughout the washing process of isoantibodies present in the donor plasma and effective against the blood of the recipient. So unlikely did this seem that we had not considered it at the time of the above mentioned transfusions, but, secure in the realization that no foreign serum would reach the recipients, had omitted compatibility tests of them and the donors. Now an experiment was undertaken to determine whether normal isoagglutinins become fixed on the possessor's own cells when these latter are allowed to sediment out of citrated plasma in the cold. Bloods suitable for the work were obtained with some difficulty owing to the fact that isoantibodies in normal rabbits are weak and rather infrequent.[9]

Experiment 1.—By the examination of mixtures of whole citrated bloods five rabbits possessing isoagglutinins were selected. In mixtures of three parts of serum separated from the clot at 37°C. and one part of 5 per cent suspension in salt solution of the susceptible cells, definite clumping occurred in all cases at room temperature. Now portions of blood from the five animals were treated to the same slow, repeated washing by sedimentation in the ice box as in the case of the donors in the transfusion experiments with washed cells already described;

[9] Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *J. Med. Research*, 1913–14, xxviii, 141.

and after the final washings, carried out with the help of the centrifuge, each cell sediment was tested as follows for serum agglutinins which might have become fixed on the corpuscles:

All possible supernatant fluid was drawn off; Locke's solution to about one-third the quantity of the original plasma was poured on; the cells were suspended for 10 minutes at 38–40°C.; and then they were thrown down by rapid, brief centrifugation. The salt solution was then immediately pipetted away and mixed in various proportions with 5 per cent suspensions of the cells that had originally been agglutinated by the plasma. As a control similar mixtures were made of cell suspensions and serum from the five animals, all kept from the time the original citrated specimens were drawn. The control serum had been removed from the clot after a few hours at incubator temperature and preserved in the ice box until needed.

In every case the salt solution clumped the cells acted upon by the control serum, while comparative tests in graded dilution showed that practically the entire original isoagglutinin content of the serum had passed into it.

The point disclosed by this experiment was most unexpected and of considerable theoretical interest. It is evident that normal isoagglutinins of the rabbit may become fixed upon the animal's own cells in the cold, to be given up again into salt solution at 37°C. Possibly in our transfusions with cells washed by sedimentation in the cold some isoagglutinins were thus carried over into the recipient. But this does not warrant the conclusion that an introduction of such isoagglutinins is the explanation of the clumping previously interpreted as autoagglutination. In the transfusions with whole citrated blood from compatible donors which resulted in what seemed to be autoagglutination the total amount of plasma injected into the recipient prior to the development of the clumping phenomenon was often considerably less and never very much more than the animal itself had to start with. Now granting that all of the alien plasma save the antibodies was removed from circulation, this would mean that the latter were left in about their original concentration in the donors, and at this concentration they had not been demonstrable *in vitro* when the donors were chosen. They cannot, then, have been responsible for the autoagglutination now seen in the recipient's shed blood and in suspensions of his preserved cells mixed with fresh serum. Nevertheless, transfusion experiments with cells washed in the warm were planned, but before they had been undertaken a new fact came to light. The corpuscles of normal rabbits

having no demonstrable isoagglutinins in the plasma were themselves found to contain such antibodies in considerable amount.

Intracellular Agglutinins.

Klein[10] in 1902 claimed that extracts of red cells in salt solution or water showed agglutinative properties. His findings have met no general recognition, perhaps in part because of their conflict with the accepted rule that circulating antibodies are confined to the plasma, but also doubtless because of the irregularity of his results and the fact that the method used to obtain them is open to criticism. To obtain the agglutinins he ground the corpuscles with quartz sand; and salt solution ground with sand becomes endowed with the ability to cause a non-specific agglutination as we have repeatedly found. In the following experiment the cells were broken up by another means.

Experiment 2.—Three rabbits with plasmas that entirely failed to clump the cells of certain other rabbits in 9 to 1 and 1 to 9 mixtures of the whole bloods were bled 5 cc. each into 10 cc. of isotonic sodium citrate solution at 37°C. The corpuscles were at once packed with the centrifuge in a room maintained at body heat, and the supernatant fluid pipetted away as completely as possible, while still warm. The temperature of the room tended to go slightly above 38°C. because of the heating up of the centrifuge.

Now portions of each specimen of sedimented red cells were spread in thin layer on watch-glasses and dried in a current of air, a process requiring about 20 minutes. To the dried material of 0.7 to 1.0 cc. of cells, 1.0 to 1.7 cc. of doubly distilled water was added; and after the resulting thin paste had stood for a few minutes, it was centrifuged; the clear, deep red, supernatant fluid pipetted off; and to ten parts of this last one part of 9.0 per cent sodium chloride solution was added to restore approximate isotonicity. The fluid remained clear. It was now used in agglutination tests with corpuscles of the sort that had remained unagglutinated by the plasmas of the animals from which the cells for drying had come. Nine parts of the extract were used to one of a 50 per cent suspension of corpuscles in salt solution. In every case there was an almost immediate clumping of the cells into coarse flocculi. Under the microscope the clumping was found to be a characteristic agglutination, with a candy-like stringing out of the adherent corpuscles when pressure was made on the cover-glass, and a resumption of the original form when pressure was relieved. There was no crenation of the cells. The flocculi failed to break up when salt solution was added in quantity at room temperature, but fell apart immediately into the component cells when warmed to 37°C. in salt solution or the original cell extract; and in the latter at once reformed on cooling to or below 34.5°C.

[10] Klein, A., *Wien. klin. Woch.*, 1902, xv, 413.

A closer study of the intracellular agglutinins of rabbits such as were here found should have considerable interest. We have examined the corpuscles of eight human bloods for similar principles, according to the method described, but the findings were negative.

DISCUSSION.

With the demonstration that rabbit red cells contain a principle capable of agglutinating characteristically the corpuscles of other rabbits, our study of the autoagglutination induced by transfusion came to an end. In brief, we had found that the remarkable clumping of the cells in the shed blood of repeatedly transfused rabbits is due in most instances to the action of isoagglutinins developing in the recipient and effective upon the alien elements circulating amongst its own cells. Interagglutination of the donor cells by the plasmas introduced with them may sometimes play a subsidiary part in the phenomenon, as may possibly agglutination of the recipient's corpuscles by weak antibodies appearing in the donor's plasma during the course of the experiment. There remain instances in which, with all such causes of clumping ruled out, the recipient's corpuscles, taken prior to transfusion and preserved *in vitro*, are agglutinated by the animal's own serum obtained after several injections of alien blood. That these are genuine instances of induced autoagglutination remains uncertain because the possibility was not excluded that intracellular agglutinins derived from the donors underwent liberation in the recipient. The best evidence for a true autoagglutination lies in the not infrequent long persistence of the antibodies in the recipient's blood. In one such instance the clumping phenomenon remained well marked for at least 133 days after the last transfusion. During this period the plethora consequent on the introduction of the blood had been quickly succeeded by a profound anemia during which the hemoglobin fell to 27 per cent (Palmer), and this in turn was followed by a rapid recovery to normal and many weeks of good health.[3] Here the destruction and replacement of the circulating elements gave assurance that whatever the original source of the agglutinin, it was directed against the rabbit's own cells. An antibody of foreign origin would scarcely have remained so long in the blood stream. Tests with serum taken from other recipients when

the clumping phenomenon was at its height, and preserved in the ice box, reinforce such findings. Thus, for example, it was observed that cells obtained from a recipient 86 days after the last transfusion, with an intervening plethora-anemia-recovery sequence, were agglutinated by serum from the animal preserved all this while. There are several facts which speak for the view that true autoagglutinins were induced in the transfused rabbits as a by-product to isoagglutinins. Our experiment in which normal isoagglutinins became fixed in the cold on their possessor's own cells shows how close is the antigenic relationship between the erythrocytes of different rabbits. And Ottenberg and Thalheimer[11] have made the significant observation with regard to the normal autoagglutinins of cats that: "Those strongly agglutinative sera which affect the red cells of a large number of other animals are usually those which are auto-agglutinative." The fact that the normal isoantibodies of cats are weak at most, while isohemolysins are entirely absent from the plasma would seem to indicate that, as in rabbits, the antigenic differentiation of the red cells is but slight. Numerous recent authors have noted that immunization with a single antigen may cause the development of antibodies of rather wide application. One need only suppose for the present case that this widening of application is such that the antibodies find a mark in the animal's own cells, as was Ehrlich's original conception.

The clumping observed in the shed blood of transfused rabbits never fails to bring together practically all of the cells. Yet our analysis of the content of the sera has shown in a number of instances that agglutinins were present only for certain cells in the circulating mixture. The apparent contradiction here seen is explained by the distribution of the susceptible cells within rouleaux. These latter, which fail to break up save when agglutination is strong, are brought together with the cells. If they have been broken up by washing with warm salt solution, and the cells dispersed, the agglutination becomes selective. Whenever the clumping of the shed blood is strong enough to disorganize the rouleaux and mass all the cells confusedly, agglutinins for all are regularly found to be present on serum analysis.

[11] Ottenberg, R., and Thalheimer, W., *J. Med. Research*, 1915–16, xxxiii, 213.

The fact that repeated subcutaneous or intraperitoneal injections with a compatible blood will cause the development of antibodies effective against such blood has been generally accepted since Ehrlich's study of induced isolysins in goats; but it has failed of recognition in its important practical bearing on the outcome of repeated transfusions in man. Our experience with rabbits repeatedly injected from compatible donors, especially such animals as showed a sudden and great blood destruction, serves to illustrate in little what must not infrequently occur in human beings. It follows that the most careful blood tests are called for with patients repeatedly retransfused at short intervals. In special, a thick spread of the patient's blood should be examined for autoagglutination which is often, as we have shown, an evidence that newly developed isoagglutinins are in circulation. Throughout the tests the temperature factor should be carefully controlled. Serum separated from the clot at 37°C. will often yield antibodies not demonstrable in that taken in the cold, and conversely agglutination mixtures examined at room temperature are far more likely to yield positive findings than those kept at blood heat.

Whether massive transfusions are preferable in pernicious anemia to repeated small ones remains uncertain. Sudden large increments of blood tend to lessen the reparative activity of the bone marrow.[4] But, on the other hand, they may also act to prevent fulminant destruction of the introduced blood by the recipient's serum antibodies. In the work already mentioned on experimental hemosiderosis in rabbits[2] some very large transfusions were given to animals that had developed strong serum antibodies, in the hope that abundant and continued blood destruction would ensue. This was never the case. The amount of antigen introduced so greatly exceeded that of the circulating antibodies that these latter failed to be destructive and plethora was maintained. It is a curious fact that in animals transfused 6 days out of every 7 during a period of 6 months the agglutinins gradually and completely disappeared, although following the first few injections they were often strong. A similar disappearance of precipitins upon long immunization has been recorded by Tschistowitsch and Nuttall.[12] Yet the transfused blood is somehow destroyed with great rapidity in animals frequently injected.

[12] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904, 127.

SUMMARY.

The massive agglutination observable in the shed blood of transfused rabbits, and associated not infrequently with sudden marked blood destruction, has a practical significance in connection with the untoward results of repeated transfusion from donors originally compatible; and it has special theoretical interest because the clumping of the cells is apparently an autoagglutination. To determine the actual source of the antibodies has been the object of the present work.

The agglutination in its most marked form has been traced to isoantibodies elicited by the presence in the body of corpuscles originally found compatible; and the frequently associated, rapid blood destruction is doubtless of similar origin. Occasionally antibodies develop in the donor bloods during the period of transfusion, but they are so weak as to be negligible. There remain instances of what would seem to be true autoagglutination due to serum bodies induced by the transfusions as a by-product, so to speak, in the manufacture of isoagglutinins. The antigenic relationship between the red cells of different rabbits is so close that normal isoagglutinins became fixed in the cold upon their elaborator's own corpuscles.

Agglutinins exist within the red cells of rabbits—as has been claimed by Klein. They are readily demonstrable in watery extracts of the dried corpuscles. Whether similar agglutinins ever exist within human cells remains to be determined. We have not found them in the normal corpuscles.

STUDIES ON THE PNEUMONIC EXUDATE.

V. The Relation of Pneumonic Lung Protease Activity to Hydrogen Ion Concentration, and a Consideration of the Origin of the Enzyme.

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(Received for publication, August 26, 1921.)

That leucocytes contain an enzyme or enzymes capable of splitting native proteins to simpler nitrogenous compounds is an accepted fact at the present time. As early as 1877 Filehne[1] succeeded in obtaining from the sputum of a case of lung gangrene by extraction with glycerol an enzyme capable of dissolving fibrin and coagulated egg white in slightly alkaline media. A few years later Stolnikow[2] and Escherich[3] obtained similar results with extracts of sputa from cases of bronchitis, pneumonia, and phthisis. At that time the origin of the enzyme was disputed.

Müller[4] conducted a series of experiments relative to the action of empyema and abscess pus on fibrin and coagulated egg white, and found that the substances were readily dissolved. The sputa from cases of lobar pneumonia at the time of or just following crisis had a similar effect; whereas, before the crisis no enzyme action could be demonstrated. He also noted that pieces of pneumonic lung immersed in toluene water and placed in the incubator for several days became considerably lighter. He was of the opinion that the enzyme came from the destroyed white blood corpuscles. Simon,[5] following out Müller's views, studied chemically the autolysis of toluene water extracts of lungs from cases of lobar pneumonia. The extracts of five out of the six cases studied gave a primary acid reaction, and were subsequently neutralized or made slightly alkaline with calcium or sodium carbonate. After incubation for 5 to 8 days the average decrease in coagulable nitrogen, or increase in non-coagulable nitrogen, was 30 per cent; and an average of 30 per cent of the increase in non-coagulable nitrogen was accounted for by an

[1] Filehne, W., *Sitzungsber. physik.-med. Soc. Erlangen*, 1877, ix, 169, cited by Stolnikow.[2]

[2] Stolnikow, J., *St. Petersb. med. Woch.*, 1878, iii, 160.

[3] Escherich, T., *Deutsch. Arch. klin. Med.*, 1885, xxxvii, 196.

[4] Müller, F., *Verhandl. Cong. inn. Med.*, 1902, xx, 192.

[5] Simon, O., *Deutsch. Arch. klin. Med.*, 1901, lxx, 604.

actual increase in albumoses. The lung of the sixth case was in the stage of red hepatization; the extract was alkaline in reaction and underwent no autolysis. Control experiments on normal lung extracts showed no autolysis. About the same time Achalme[6] working with pus from various localities demonstrated the presence of a proteolytic enzyme capable of digesting fibrin and casein. He, also, concluded that this enzyme was derived from the leucocytes.

More recently Opie[7] has carefully studied the proteolytic enzymes of sterile inflammatory exudates produced by intrapleural injections of an aleuronat suspension in rabbits and dogs. He concluded that there were two distinct proteolytic enzymes present in the exudates—a leucoprotease derived from the polymorphonuclear leucocytes and acting best in slightly alkaline media (0.2 per cent sodium bicarbonate), and a lymphoprotease derived from the mononuclear cells and acting best in slightly acid media (0.2 per cent acetic acid). The action of both enzymes was inhibited by the antiferment of normal beef serum. Jobling and Strouse[8] repeated some of Opie's work and came to essentially the same conclusions except that they believed there was also an erepsin-like enzyme present, active in both acid (0.2 per acetic acid) and alkaline (0.2 per cent sodium bicarbonate) media. Dernby[9] demonstrated in leucocytic suspensions enzymes capable of splitting peptone (optimum pH 7.6 to 8.0).

In a series of investigations on pneumonic exudates undertaken in this laboratory Lord[10] with cellular suspensions from pneumonic lungs ranging from pH 3.1 to 7.3 obtained visible erosion on Löffler's blood serum only at the higher end of the scale (pH 6.7 to 7.3). Peptone solutions were appreciably split by these cellular suspensions, and most complete splitting occurred at pH 5.2 or 6.3. He formulated the hypothesis that during the course of the disease in the pneumonic lung there is a gradual decrease in pH or increase in acidity. Primarily and at that time when the reaction of the exudate is about that of the circulating blood (pH 7.3 to 7.5) there is a digestion of the more highly organized proteins (fibrin, serum albumin, etc.) following the liberation of enzyme through cellular disintegration. With subsequent increase in acidity (beyond pH 6.7) the activity of this protease diminishes and conditions reach an optimum for the activity of the peptone-splitting enzyme. This peptonase or ereptase carries the splitting of the digestion products to amino-acids, absorbable as such; and resolution takes place.

It seemed advisable to determine quantitatively the digestive action of the exudate on one of the higher proteins and the relation of hydrogen ion concentration to the activity of the enzyme. As a

[6] Achalme, *Compt. rend. Soc. biol.*, 1899, li, 568.
[7] Opie, E. L., *J. Exp. Med.*, 1906, viii, 410.
[8] Jobling, J. W., and Strouse, S., *J. Exp. Med.*, 1912, xvi, 269.
[9] Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.
[10] Lord, F. T., *J. Exp. Med.*, 1919, xxx, 379.

substrate fibrin was chosen, since it is one of the higher proteins which must be broken down in the pneumonic lung during the process of resolution. Since a cellular suspension of the consolidated lung, rather than of the exudate alone, was used, it was necessary to rule out the action of enzymes contained in normal lung tissue.

EXPERIMENTAL.

1. Preparation of Substrate.—A mass of fresh horse fibrin about the size of a baseball was picked and washed practically free from adhering blood clot, and then passed through a meat chopper with a nut butter cutter attachment. The mash was centrifuged and washed repeatedly with water until the supernatant fluid was clear and colorless. The water was filtered off and the fibrin dried at 100°C. over night on a steam water bath. After grinding, the yield was 2.7 gm.

2. Preparation of Enzymes.—Portions of gray-red hepatization and normal lung from a case of Type I lobar pneumonia had each been previously ground in a meat chopper with a nut butter cutter attachment and kept in the ice box several weeks following the addition of chloroform and toluene. The consolidated lung mash was washed through two thicknesses of sterile gauze with 0.85 per cent sterile salt solution and the washings were centrifuged and washed four or five times with salt solution. The final centrifuging (No. 7 stop for 7 minutes) yielded 5 cc. of "cells," which were made up to 15 cc. with sterile salt solution. The normal lung mash was ground with sterile sand in a sterile mortar; and, after separating the sand from the suspension by centrifuging at low speed, treated as above. The yield was only 0.6 cc. of "cells;" these were made to 15 cc. with salt solution.

3. Method.—To dry 60 cc. sterile flasks were added phosphate mixtures, fibrin, 10 cc. of sterile water (Nos. 6 to 15 inclusive, 15 cc.), and 1.0 cc. of cellular suspensions (Nos. 6 to 15 inclusive, 1.5 cc.) (Table I). To those flasks intended to have pH values of 4.0 and 5.0 were added 4 drops of a 2 per cent aqueous solution of sodium alizarin sulfonate, and to the remainder 2 drops of a 0.01 per cent solution of phenol red. The flasks were adjusted approximately to the desired pH by the addition of normal and 0.1 normal hydrochloric acid and sodium hydroxide and comparison with a set of standard pH solutions prepared according to Clark and Lubs.[11] Water was added to make the total volume 20 cc. (Nos. 6 to 15 inclusive, 30 cc.). After thorough agitation 10 cc. samples were removed from Flasks 6 to 15 inclusive. 1 cc. of toluene was added to each flask and all were tightly corked and placed in the incubator at 37.5°C. for 5 days.

Cultures taken after 12 hours incubation and at the end of the experiment were sterile. During incubation there was no appreciable change in reaction.

The coagulable proteins were precipitated by the addition of trichloroacetic acid (3 per cent of total volume) according to Greenwald.[12] The tubes were

[11] Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.

[12] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

TABLE I.

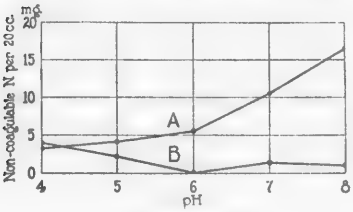
The Composition of the Various Digestion Mixtures and the Results of the Determinations.

Tube No.	Fibrin.	Pneumonic lung cellular suspension.	Normal lung cellular suspension.	0.5 M KH_2PO_4.	0.5 M $Na_2HPO_4 \cdot 2H_2O$.	Water, acid, and alkali.	pH	Non-coagulable nitrogen per 20 cc.			Amino nitrogen per 20 cc.		$\frac{\text{Amino nitrogen}}{\text{Non-coagulable nitrogen}} \times 100$.
								Before digestion.	After digestion.	Actual digestion.	After digestion.	Actual digestion.	
	gm.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	0.30*			2.0		18.0	4.0	—	1.9	—	0.46	—	
2	0.30			1.8	0.2	18.0	5.0	—	2.5	—	0.50	—	
3	0.30			1.6	0.4	18.0	6.0	—	4.2	—	0.52	—	
4	0.30			0.4	1.6	18.0	7.0	—	5.4	—	0.62	—	
5	0.30				2.0	18.0	8.0	—	6.3	—	0.74	—	
6		1.5		3.0		25.5	4.0	1.0	1.2	0.2	0.24	—	
7		1.5		2.7	0.3	25.5	5.0	0.6	1.0	0.4	0.28	—	
8		1.5		2.4	0.6	25.5	6.0	0.8	1.7	0.9	0.34	—	
9		1.5		0.6	2.4	25.5	7.0	0.5	2.0	1.5	0.54	—	
10		1.5			3.0	25.5	8.0	0.7	2.5	1.8	0.56	—	
11			1.5	3.0		25.5	4.0	0.1	0.3	0.2	—	—	
12			1.5	2.7	0.3	25.5	5.0	0.1	Lost.	?	—	—	
13			1.5	2.4	0.6	25.5	6.0	0.2	0.8	0.6	—	—	
14			1.5	0.6	2.4	25.5	7.0	0.2	0.3	0.1	—	—	
15			1.5		3.0	25.5	8.0	0.2	0.6	0.4	—	—	
16	0.30	1.0		2.0		17.0	4.0	—	5.8	2.7	1.02	0.32	12
17	0.30	1.0		1.8	0.2	17.0	5.0	—	7.7	4.2	1.22	0.44	11
18	0.30	1.0		1.6	0.4	17.0	6.0	—	11.3	5.4	1.58	0.72	13
19	0.30	1.0		0.4	1.6	17.0	7.0	—	17.9	10.5	2.76	1.60	15
20	0.30	1.0			2.0	17.0	8.0	—	25.2	16.4	3.26	1.96	12
21	0.30		1.0	2.0		17.0	4.0	—	6.2	4.0	—	—	
22	0.30		1.0	1.8	0.2	17.0	5.0	—	4.6	1.6†	—	—	
23	0.30		1.0	1.6	0.4	17.0	6.0	—	5.0	0.0	—	—	
24	0.30		1.0	0.4	1.6	17.0	7.0	—	7.1	1.4	—	—	
25	0.30		1.0		2.0	17.0	8.0	—	7.8	0.9	—	—	

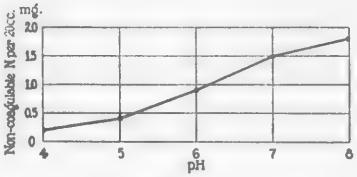
* Total nitrogen determination 0.042 gm.

† Estimating No. 12 flask after digestion as 0.5 mg.

stoppered, shaken, and allowed to stand 12 to 15 hours in the ice box before filtering. Nitrogen determinations on the filtrates were made by Folin's[13] macro Kjeldahl method, using 5 cc. samples and 5 cc. of his digestion mixture. Duplicate determinations were made on all filtrates. The non-coagulable nitrogen representing the actual digestion of the fibrin by the cellular enzymes was obtained by subtracting the sum of the non-coagulable nitrogen of the fibrin and lung controls from that of the digestion mixture. The results are expressed as the average amount of non-coagulable nitrogen present in 20 cc. Amino nitrogen was determined by Van Slyke's[14] nitrous acid method using 2 cc. samples of the filtrates following the precipitation of the coagulable nitrogen. Blank determinations made with distilled water and with distilled water saturated with toluene eliminated the presence of traces of the preservative as a source of error. Results are expressed as the amount of amino-acid nitrogen present in 20 cc.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Proteolytic activity of pneumonic (*A*) and normal (*B*) lung cellular suspensions at varying hydrogen ion concentrations.

TEXT-FIG. 2. Autolysis of pneumonic lung cellular suspensions at varying hydrogen ion concentrations.

The results clearly show (Text-fig. 1) that there is present in the pneumonic lung an enzyme, or enzymes, capable of digesting horse fibrin. Similar results were obtained with cellular suspensions from the consolidated lungs of lobar pneumonias other than those caused by Type I pneumococcus. In the range of reactions chosen as physiological possibilities the enzyme shows the greatest activity at the most alkaline end of the scale (pH 8.0). At this hydrogen ion concentration it was able to convert 46 per cent[15] of the fibrin origi-

[13] Folin, O., and Wright, L. E., *J. Biol. Chem.*, 1919, xxxviii, 461.

[14] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

[15] Calculated from the following figures (Table I): total nitrogen of fibrin, 42 mg.; non-coagulable nitrogen of fibrin at pH 8.0, 6.3 mg.; and actual increase in non-coagulable nitrogen in digestion mixture at pH 8.0, 16.4 mg.

nally present to a non-coagulable form after 5 days incubation. The digestion at pH 4.0 is comparatively slight; and there is a gradual increase of digestion corresponding to decrease in acidity. Appreciable autolysis of the pneumonic lung controls occurred, slight at pH 4.0 and 5.0 with a gradual increase towards the alkaline side (Text-fig. 2). Presumably the same enzyme is responsible for both digestive processes. The amino-acid nitrogen determinations show that in the pH 7.0 digestion mixture the protein splitting was most complete. This corresponds roughly to the optimum hydrogen ion concentration of the pneumonic lung peptonases or ereptases, as reported by Lord[10] and confirmed in a paper to follow. The cellular suspension controls of normal lung show an appreciable digestion of the fibrin at pH 4.0, and practically no increase in non-coagulable nitrogen in the less acid media (Text-fig. 1).

DISCUSSION.

This protease can be conceived as arising from one or more of four sources: (*a*) the pneumococci, (*b*) the cells present in the normal lung, (*c*) the blood serum, and (*d*) the cells of the pneumonic exudate.

Avery and Cullen[16] have been able to obtain from pneumococci intracellular enzymes capable of digesting fibrin. With fibrin freshly prepared from rabbit blood and the bile extract from the organisms contained in 200 cc. of a plain bouillon culture of Pneumococcus Type II, the total actual digestion at pH 7.4 amounted to an increase of 0.26 mg. of amino nitrogen. With 0.2 gm. of commercial fibrin and a similar amount of enzyme, their actual increase in amino nitrogen was only 0.11 mg. Furthermore, since their enzyme contained non-coagulable nitrogen and in addition underwent autolysis on incubation, it is conceivable that in their digestion mixtures a portion of the increase in amino nitrogen might have been due not to an enzyme digesting native protein but to one autolyzing the enzymatic non-coagulable nitrogen—a peptonase, the presence of which they clearly demonstrated in extracts of the pneumococcus. With 0.3 gm. of fibrin and 0.33 cc. of washed cellular suspension from the pneumonic lung the total increase in amino nitrogen at pH 7.4 fell somewhere between 1.60 mg. (pH 7.0) and 1.97 mg. (pH 8.0). Stained

[16] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 547.

smears of the cellular suspension showed no intact organisms. In the washing of the cellular material in preparation one would expect the enzyme liberated from the disintegrated pneumococci to have been lost. Undoubtedly the pneumococcus protease contained in 0.33 cc. of washed cellular suspension is negligible when considering digestion amounting to 1.60 to 1.97 mg. of amino-acid nitrogen.

Opie[7] has shown that sterile pleural exudates containing an excess of mononuclear cells exhibit maximum proteolytic activity in slightly acid media (0.2 per cent acetic acid). The phagocytic mononuclear or endothelial cells are derived from the endothelial cells lining the blood and lymph vessels.[17] A cellular suspension obtained from a normal lung, or to a less degree from the true lung substance of a pneumonic lung, would contain, so far as those cells which are known to contain proteolytic enzymes are concerned, chiefly endothelial cells. Consequently from Opie's work one would expect maximum digestion in a slightly acid medium. This was exactly what happened in the normal lung cellular suspension and fibrin digestion mixtures. Since nearly twice as much fibrin was digested at pH 4.0 by the 0.04 cc. of normal lung cellular suspension as by the 0.33 cc. of pneumonic cellular suspension, it can be safely assumed that the latter did not contain as much true lung substance as the former. In spite of the presence of at least an equal amount of true lung substance, the normal lung suspension as compared with that of the pneumonic lung showed but slight digestion in the slightly acid, neutral, and slightly alkaline digestion mixtures. Hence, the enzyme, or enzymes, derived from the true lung substance of a pneumonic lung play but a slight rôle in the proteolysis of fibrin by the pneumonic lung cellular suspension except in moderately acid media (pH 4.0).

Proteases or trypsin-like ferments are known to be normally present in blood serum; but they are held in abeyance by the presence of antitrypsin. Delezenne and Pozerski[18] observed that the antitryptic power of the serum is lost following extraction with chloroform. It is conceivable that in lung cellular suspensions preserved with chloroform and toluene the serum proteases become potentially

[17] Mallory, F. B., The principles of pathologic histology, Philadelphia and London, 1918.

[18] Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, lv, 327, 690.

active. However, frequent washings in preparation of the enzyme previous to the experiment would certainly remove them.

The protease of pneumonic lung cellular suspensions is derived chiefly from the leucocytes of the exudate. The other possible sources play but a minor rôle. Confirming the work of many other investigators, it was found that this enzyme is most active in a slightly alkaline medium (pH 8.0).

SUMMARY.

1. Washed cellular suspensions of pneumonic lungs, previously preserved with chloroform and toluene, contain a protease or proteolytic ferment, derived chiefly from the leucocytes of the exudate.

2. This protease is able to convert horse fibrin to a non-coagulable split product. In a pH range of 4.0 to 8.0 the digestion is slight at the most acid end. With decrease in acidity there is a gradual rise in activity to pH 6.0, and then a sharp increase up to the maximum digestion at pH 8.0. Judging from the amino nitrogen determinations, the most complete splitting occurs at pH 7.0. The degree of autolysis occurring in the pneumonic lung cellular suspension controls suggests that the active enzyme is identical with that causing the digestion of the fibrin.

3. Washed cellular suspensions of normal lungs, previously preserved with chloroform and toluene, contain a proteolytic enzyme which is active in a moderately acid medium (pH 4.0) and essentially inactive in less acid, neutral, and slightly alkaline media.

The author is indebted to Dr. F. T. Lord for many helpful suggestions.

THE TRANSMISSION OF AGGLUTININS OF BACILLUS ABORTUS FROM COW TO CALF IN THE COLOSTRUM.

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(Received for publication, September 2, 1921.)

In the work on infectious abortion associated with *Bacillus abortus* carried on in this Department since 1917,[1,2] the blood serum and transudates of fetuses have been tested from time to time to determine

TABLE I.

Agglutinin Content of Blood of Dam and Aborted Fetus.

Case No.	Blood serum.		*B. abortus* isolated.*	Remarks.
	Dam.	Fetus.		
493	1:1,280	1:40	Yes.	
374	1:1,280	1:80	"	1:20 to 1:80 show the same degrees of agglutination. Abdominal and thoracic transudates negative.
652	1:1,280	0	"	
506	?	0	"	
270	1:1,280	0	"	
590	1:320	1:40	"	
595	1:320	1:20	"	
579	1:80	1:20	No.	
509	?	1:40	Yes.	
518	1:1,280	1:20	"	
668	1:320	1:20	"	Colostrom 1:2,560.
671	1:1,280	1:20	"	" 1:2,560.

* From autopsy records of the Department.

the relation between the concentration of agglutinins in the blood serum of mother and fetus. The results, given in Table I, indicate that even when the serum of the mother has a high agglutinin content,

[1] Smith, Theobald, *J. Exp. Med.*, 1919, xxx, 325.

[2] Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx 341

little or none is found in the fetal blood. The agglutinin figures given represent the agglutination limits of series of dilutions.

Tests of the blood of calves within the first few days of life presented different results. In some a relatively high titer was observed. The variation from case to case was finally explained by the absorption of agglutinins in the colostrum, as the experiments reported below amply demonstrate.

The literature on the transmission of various antibodies in the milk is fairly rich. Only a few publications bear more directly on the subject in hand. For a thorough review up to 1912 the reader is referred to Famulener's article.[3] In his own investigations on this subject, Famulener immunized goats with sheep blood corpuscles. He tested the antibody content of the mother's serum and colostrum at time of parturition and the blood of the kids before and after they had ingested colostrum. He found that in immunized goats the colostrum was rich in antibodies but in the kids no antibodies were present until after taking the colostrum. He observed that the titer of the colostrum in many cases was above that of the mother's serum, and that the milk quickly dropped in antibody content. From his experiments he concluded that the hemolytic antibodies in the young are received through the colostrum and absorbed by the young from the gastrointestinal tract, but that this absorption can only take place during the first few days after birth.

Rettger and White[4] in 1918 and Rettger[5] in 1920 made the statement that, without exception, cows with a positive agglutination to *B. abortus* gave birth to calves which showed the same reaction. This statement is true only after the first feeding with colostrum.

Recently Reymann[6] in studying the transfer of normal agglutinins from mother to young also worked with goats, but he brings out no new points concerning the transmission of agglutinins. Strangely enough he fails to refer to Famulener's results.

EXPERIMENTAL WORK.

The experiments to be reported differ from those of others in that they deal with the naturally acquired and stored agglutinins as the result of a definite infectious disease of cows, localized in the fetal membranes. Earlier experiments deal only with agglutinins experimentally induced.

[3] Famulener, L. W., *J. Infect. Dis.*, 1912, x, 332.

[4] Rettger, L. F., and White, G. C., *Storrs Agric. Exp. Station, Bull. 93*, 1918, 225.

[5] Rettger, L. F., *Abstr. Bact.*, 1920, iv, 13.

[6] Reymann, G. C., *J. Immunol.*, 1920, v, 227.

The procedure for the collection and dilution of the blood serum for the agglutination tests is the same as described in a previous paper.[2] The milk was handled as follows:

The samples were centrifuged and the cream layer was removed. This was necessary in order to obtain a clear whey; however, with some samples of colostrum which were very thick the centrifuging was omitted. The samples were then warmed to about 35°C. and several drops (8 to 10) of a solution of a rennet tablet in sterile distilled water were added. The tubes were well shaken and left standing in the water bath 15 to 20 minutes. By that time a firm coagulum had formed. In order to separate the whey quickly, the tubes were then put into warmer water, at about 45°C. This caused the coagulum to shrink and a clear whey to separate. This was drawn off and used for the agglutination tests. Frequent tests have shown that the rennet does not influence the agglutinins present.

The preparation and standardization of the antigen for blood and milk was as follows:

Bacillus abortus was grown on agar at 37°C. for 24 to 48 hours. The growth was washed off in 0.85 per cent salt solution. This was standardized to a given opacity by means of the Gates instrument.[7] A depth of disappearance of 2.4 cm. was taken as the standard. Each lot of antigen was made up to this density before use.

In Table II the agglutination titers of the blood and colostrum of seven cows and their calves soon after birth are given to illustrate the conditions usually found. Of these offspring, three (Nos. 488, 619, and 484A) were tested after suckling and agglutinins found in their blood. Two were negative when tested before suckling (Nos. 484B and 625). Of two premature calves, one had agglutinins in the blood (No. 567), the other not (No. 483).

These random illustrations showed the need of determining by suitably controlled tests the relation of the ingestion of colostrum to the agglutinin titer. In Table III illustrations of such tests are given. In the first case agglutinins appeared in the blood of the calf after it had suckled a cow (not its mother) whose colostrum had a high titer. In the second, the calf was fed colostrum artificially and agglutinins appeared but of a lower titer than in the preceding calf

[7] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

TABLE II.

Material tested.	Date of blood test.	Dilutions. 1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	Control.
Cow 484 and twin calves A and B, born Mar. 23, 1921. A had nursed before the first bleeding; B, much weaker, had not nursed until after the first bleeding.												
	1921											
Cow's blood	Mar. 23	C.*	C.	++++	+	−	−	−				−
Colostrum	" 23	"	++++	++++	++	+	−	−				−
Blood, Calf A	" 23	+++	+	+	−	−	−	−				−
" " "	" 24	C.	+++	+	+	−	−	−				−
" " B	" 23	−	−	−	−	−	−	−				−
" " "	" 24	C.	++	+	−	−	−	−				−
Cow 488 and calf which had nursed before blood test. Calf born Feb. 18, 1921.												
Cow's blood	Feb. 18	C.	C.	C.	++++	+++	++	±	−	−	−	−
Colostrum	" 18	++++	++++	++++	++++	+++	C.	C.	C.	C.	++	−
Calf's blood	" 18	++++	+++	++	++	++++	"	"	+++	±	−	−
" "	" 19	C.	+++	+	++	C.	"	"	++++	+	−	−
Cow 619 and calf which had nursed before first bleeding. Calf born June 9, 1921.												
Cow's blood	June 9	C.	C.	C.	C.	C.	C.	C.				−
Colostrum	" 9	"	"	"	"	"	"	++++	+++	++	++	−
Calf's blood	" 9	"	++++	++++	++++	++++	++++	++++				−
" "	" 10	++++	++++	C.	C.	C.	C.	C.				−

Cow 625 and calf which had not nursed before first bleeding. Calf born July 3, 1921.

Cow's blood	July 5	C.	C.	C.	C.	++	++	++				−
Calf's "	" 3	−	−	−	−	−	−	−				−
" "	" 4	C.	C.	++++	++	+	±					

Cow 483 and 8 mo. calf (premature) which had not nursed before first bleeding. Calf born Mar. 5, 1921.

Cow's blood	Mar. 5	C.	C.	C.	C.	++++	++++	+++				−
Colostrum	" 5	++++	++++	++++	++++	++++	++++	+++	++			−
Calf's blood	" 5	−	−	−	−	−	−	−				−
" "	" 6	+++	+	−	−	−	−	−				−
" "	" 7	++++	+	−	−	−	−	−				−

Cow 477 and calf which may have nursed before bleeding.

Cow's blood	May 28	C.	C.	C.	++	+	−	−				−
Colostrum	" 28	++++	++++	+++	++	+	−	−				−
Calf's blood	" 28	+	−	−	−	−	−	−				−
" "	" 31	++++	+	−	−	−	−	−				−

Cow 567 and premature unfed calf. *B. abortus* found in the organs subsequently.

Cow's blood	Feb. 16	C.	C.	C.	C.	+++	++	−				−
Colostrum	" 16	+++	++++	++++	++++	+++	+	−				−
Blood of fetus	" 16	++++	++++	+++	++	+	−	−				−

* The plus signs stand for degrees of clumping from almost complete (+ + + +) to just visible clumping (+). The minus sign means less than 1:20, and no clumping in controls. The letter C stands for complete clearing of the fluid.

TABLE III.

No. of dam.	Titer.		Age of calf when blood was tested.	Titer of blood.	Remarks.
	Blood.	Colostrum.			
Calf of Cow 627, born July 7, 1921, at 9.40 a.m. Restrained from taking colostrum until after three examinations of the blood, then allowed to suckle a high titer cow (No. 626) which had given birth to a calf the same day.					
627	1:160	1:320	1 hr., 50 min.	—	
			7 hrs., 10 "	—	Calf received 1 quart of milk free from agglutinins.
			21 " 30 "	—	
	Calf placed with Cow 626.				
626	1:1,280	1:2,560	32 hrs.	1:640	
			46 " 50 min.	1:640	
Calf born of a high titer cow (No. 683), July 21, 1921, at 12.30 p.m. Restrained from taking colostrum until after two examinations of blood, then fed dam's colostrum which had been refrigerated and warmed.					
683	1:1,280	1:2,560	4 hrs., 45 min.	—	Calf received 42 ounces of milk free from agglutinins.
			21 " 10 "	—	
	Calf received 36 ounces of colostrum.				
			28 hrs., 30 min.	1:40	Calf received 92 ounces of colostrum.
			45 " 10 "	1:160	
Calf born of a high titer cow (No. 634), July 12, 1921, at 11 a.m. Restrained from taking colostrum until after three blood examinations, then fed dam's colostrum which had been refrigerated and warmed.					
634	1:320	1:640	1 hr.	—	
			6 hrs.	—	Calf received 1 quart of milk free from agglutinins.
			22 " 30 min.	—	
	Calf received 33 ounces of colostrum.				
			29 hrs.	—	
			45 "	—	
Calf born of a high titer cow (No. 657), Sept. 16, 1921, at 4.20 p.m. Restrained from taking colostrum or milk until after two examinations of the blood, then fed milk free from agglutinins.					
657	1:1,280	1:1,280	40 min.	—	
			16 hrs., 40 "	—	
	Calf received milk.				
			2 days, 16 hrs., 40 min.	—	
			5 " 16 " 40 "	—	

TABLE III—*Concluded.*

No. of dam.	Titer.		Age of calf when blood was tested.	Titer of blood.	Remarks.
	Blood.	Colostrum.			
Calf born of a low titer cow (No. 653), July 3, 1921, at 2 p.m. Restrained from taking colostrum until after one examination of the blood, then allowed to suckle a high titer cow (No. 654) which had given birth to a calf the same day. Milk was collected from Cow 653, 4 days after parturition, and blood 25 days after.					
653	1:20	1:160	1 hr., 35 min.	—	
	1:20	—(milk)			
		Calf placed with Cow 654.			
654	1:320	1:1,280	18 hrs., 50 min.	1:320	
Calf born of a high titer cow (No. 655), July 23, 1921, at 5.30 a.m. Restrained from suckling until after one blood examination, then allowed to suckle dam.					
655	1:640	1:1,280	5 hrs., 5 min.	—	
		Calf placed with Cow 655.			
			11 hrs., 20 min.	1:160	
			32 " 10 "	1:640	
Calf born of a high titer cow (No. 684), July 22, 1921, at 11.30 a.m. Restrained from suckling until after two blood examinations, then allowed to suckle dam.					
684	1:640	1:1,280	2 hrs., 15 min.	—	
			6 " 10 "	—	
		Calf placed with Cow 684.			
			11 hrs., 50 min.	1:160	
			23 " 15 "	1:320	

which suckled naturally. In the third illustration, the calf although fed colostrum failed to show agglutinins in its blood. In the fourth case, the colostrum of the mother had a high titer but the calf's blood was negative and remained so for nearly 6 days while the colostrum was being withheld. Later tests were interfered with by the death of the calf. The remaining cases reenforce the data given in the others. In the seventh, the agglutinins in the blood of the calf rose quite rapidly from the 11th to the 23rd hour.

In Table IV, the results of agglutination tests on the blood serum and colostrum of the mother and the blood serum of the new-born calf before and after the first meal of colostrum are presented in more detail to add to the data already presented and to show certain slight

TABLE IV.

No. of dam.	Age of calf when blood was tested.	Material tested.	Dilutions. 1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Control.
Calf born of a high titer cow (No. 655), at 5.30 a.m. Restrained from suckling until after one blood examination, then allowed to suckle the dam.											
655		Cow's blood.	C.	C.	C.	++++	+++	+	−		−
655		Colostrum.	"	"	"	C.	++++	++	+		−
Calf.	5 hrs., 5 min.	Calf's blood.	−	−	−	−	−	−	−		−
		Calf allowed to suckle dam.									
"	11 " 20 "	Calf's blood.	C.	++++	++	+	−	−	−		−
"	32 " 10 "	" "	"	C.	++++	+++	+	±	−		−
Calf born of a low titer cow (No. 664), at 5.20 p.m. Restrained from suckling until after one blood examination, then allowed to suckle the dam.											
664		Cow's blood.	C.	++	+	±	−	−	−		−
664		Colostrum.	+++	+++	++	+	−	−	−		−
Calf.	20 min.	Calf's blood.	−	−	−	−	−	−	−		−
		Calf allowed to suckle dam.									
"	4 hrs.	Calf's blood.	++	+	+	−	−	−	−		−
"	6 " 10 min.	" "	++++	+++	++	+	−	−	−		−
"	14 " 30 "	" "	++++	++++	+++	++	+	−	−		−

Calf born of a high titer cow (No. 669), at 1.20 p.m. Restrained from suckling until after one blood examination, then allowed to suckle the dam.

669		Cow's blood.	C.	C.	C.	++++	+	−	−	−	−
669		Colostrum.	+++	++++	++++	++++	+++	+++	++	+	−
Calf.	35 min.	Calf's blood.	−	−	−	−	−	−	−	−	−
		Calf allowed to suckle dam.									
"	2 hrs., 40 "	Calf's blood.	+	++	+++	+	+	−	−	−	−
"	5 " 40 "	" "	++++	+++	+++	++++	+++	++	+	−	−
"	19 " 40 "	" "	++++	++++	+++	++	+++	+++	+++	++	−
"	67 " 40 "	" "	C.	C.	++++	++++	+++	++	±	−	−

Calf born of a high titer cow (No. 411), at 3.30 p.m. Restrained from suckling until after one blood examination, then allowed to suckle the dam.

411		Cow's blood.	C.	C.	C.	++++	+++	+	−	−	−
411		Colostrum.	+++	+++	++++	++++	+++	++++	++++	++++	−
Calf.	35 min.	Calf's blood.	−	−	−	−	−	−	−	−	−
		Calf allowed to suckle dam.									
"	1 hr., 50 "	Calf's blood.	++	+	−	−	−	−	−	−	−
"	3 hrs., 50 "	" "	C.	++++	++++	++++	+++	++	−	−	−
"	18 "	" "	"	++++	++++	+++	+++	+++	++	+	−
"	72 "	" "	"	++++	++++	++++	++++	+++	+	−	−

irregularities in the flocculation of *Bacillus abortus* in the series of tubes containing dilutions of the colostrum and of the blood serum of the calf. These irregularities are evident in the calf's blood serum and colostrum of No. 669 and in the colostrum of No. 411. Similar irregularities are shown in Table II (Nos. 488 and 619).

In three cases of Table IV, the agglutinin titers of blood serum and colostrum of the dam were high but that of the calf's serum was negative until after the first meal. The fourth case (No. 664) presents a lower titer but the transmission of agglutinins to the calf is nevertheless in evidence. The higher concentrations of agglutinins in the colostrum than in the blood serum of the mother is indicated in three out of the four cases. In two cases the titer of the calf's serum rose above that of the mother.

DISCUSSION.

The tabulated observations and experiments are sufficient to establish the fact that even when the blood and colostrum of the dam have a relatively high content of agglutinins towards *Bacillus abortus*, the blood of the new-born calf, with rare exceptions, is free from such antibodies until the animal has suckled and taken in the colostrum. The only exception found is the last case in Table II. In this premature calf the autopsy record shows that the stomachs were still free from food and that *Bacillus abortus* was present in the spleen, liver, and kidneys. The antibodies which appear in the calf's blood are absorbed from the digestive tract. The precise age of the calf after which agglutinins fail to enter the blood as such has not been determined. Experiments bearing on this aspect of the subject are under way.

When colostrum was withheld and milk of a low or negative agglutinin titer substituted, agglutinins failed to appear or accumulate in the blood of the calf. The longest period following birth during which the colostrum was withheld in the various tests was 5 days.

A study of the tables shows, what has been pointed out by others, that the concentration of the agglutinins in the colostrum may exceed that of the blood at and immediately after parturition.

The rate of absorption into the blood is shown to be fairly rapid. Agglutinins began to appear slightly over an hour after the calf

had ingested colostrum. They were nearly at the maximum concentration 5 hours after feeding.

The test upon all samples was carried out as shown in detail in Table IV. The dilutions were made in series from 1:20 to 1:1,280 or 1:2,560, each tube being one-half the dilution of the preceding tube of the series. By this method certain irregularities in the agglutinin concentration in the colostrum were occasionally encountered. In certain tubes of a series the flocculation was stronger than in a higher, preceding concentration. In such cases the irregularities may appear in the blood serum of the calf after taking colostrum, but the blood serum of the mother does not present them.

CONCLUSIONS.

The agglutinins towards *Bacillus abortus* found in the blood serum of new-born calves are obtained from the mother through the colostrum. Calves at birth, unfed, are without agglutinins. The problem of the production of agglutinins by the fetus in whose tissues *Bacillus abortus* has multiplied and which is subsequently expelled prematurely is not touched by these observations.

THE ERYTHROPOIETIC ACTION OF GERMANIUM DIOXIDE.

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Not only have the substances which by their commonness, such as the salts of sodium, potassium, and calcium, or by their striking and unexpected action on living tissues, such as radium and its derivatives, been intensively studied, but also many observations have been reported of the therapeutic trials and physiological action of the rarer elements. Cerium,[1] cesium,[2] erbium,[3] indium,[4] lanthanum,[5] neodmiumy,[5] palladium,[6] praseodymium,[1] rhodium,[7] rubidium,[8] ruthenium,[9] samarium,[10] selenium,[11] tellurium,[12] thallium,[13] thorium,[14] titanium,[15] vanadium,[16] yttrium,[17] and zirconium[18] have all received some attention from the physiological point of view.

[1] Frouin, A., *Compt. rend. Acad.*, 1920, clxx, 1471.
[2] Kaiser, L., *Arch. nêer. physiol.*, 1918–19, iii, 587.
[3] Mines, G. R., *J. Physiol.*, 1910, xl, p. xlviii.
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[5] Rénon, L., *Bull. et mém. Soc. méd. hôp. Paris*, 1920–21, xliv, 602.
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[7] Rémond, A., *Schweiz. Apoth. Z.*, 1919, lvii, 242.
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[9] Kalle and Co., German Patent No. 289,620, July 31, 1913.
[10] Esnault and Bron, *Bull. et mém. Soc. méd. hôp. Paris*, 1920–21, xliv, 606.
[11] Duhamel, deB.-G., *Compt. rend. Soc. biol.*, 1919, lxxxii, 724.
[12] Shie, M. D., and Deeds, F. E., *Pub. Health Rep., U. S. P. H.*, 1920, xxxv, 939.
[13] Pöhlmann, A., *Arch. Dermatol. u. Syph.*, 1912–13, cxiv, 633.
[14] Gudzent, F., and Herschfinkel, *Strahlentherap.*, 1916, vii, 519.
[15] Hanzlik, P. J., and Tarr, J., *J. Pharmacol. and Exp. Therap.*, 1920, xiv, 221.
[16] von Oefele, F., *New York Med. J.*, 1913, xcvii, 78.
[17] Mines, G. R., *J. Physiol.*, 1910, xl, 327.
[18] Hébert, A., *Bull. Soc. chim.*, 1906, xxxv, series 3, 1299.

Germanium is absent from this list. We have been unable to find in the literature any references to therapeutic or physiological studies of this element or its compounds.

EXPERIMENTAL.

The germanium dioxide used in these experiments was supplied by Müller.[19]

It was found that the mature albino rat can withstand the subcutaneous injection of 180 mg. of a 0.4 per cent solution of germanium dioxide per kilo of body weight without fatal result or the appearance of any symptoms whatsoever of a toxic effect. The detailed report of these experiments is now in press.[20] That study was planned as a preliminary to the determination of the effect of germanium on the erythropoietic system because of the interest that has been attached to arsenic in this connection and because germanium occupies a place in the periodic system next to arsenic and in many of its reactions resembles this latter element.

For this study there were used two litters of mature albino rats. In one litter, 150 days old, there were three males and four females. In the other, which was about 175 days old, there were four males and four females. The females had been segregated before puberty and had never been pregnant. All the rats had from birth been on the same varied diet which was continued throughout the investigation. We thus had for the purposes of our experiment two groups of animals of two lots each in which constitutional variability was reduced to a minimum. The relatively low variability within the litter has been shown by Jackson[21] and King.[22] Each lot of rats was kept in a separate cage.

Two preliminary determinations, 7 days apart, were made of the erythrocyte and leucocyte counts of each rat of each lot in order to establish the normal values. One rat of each lot was reserved as control. Weekly counts of the cellular blood components were made

[19] Müller, J. H., *J. Am. Chem. Soc.*, 1921, xliii, 1085.

[20] Hammett, F. S., Müller, J. H., and Nowrey, J. E., Jr., *J. Pharmacol. and Exp. Therap.*, 1922 (in press).

[21] Jackson, C. M., *Am. J. Anat.*, 1913–14, xv, 1.

[22] King, H. D., *Anat. Rec.*, 1915, ix, 751.

of all the rats—controls and tests. The blood on which the counts were made was obtained by cutting off a bit of the tail for each determination. The blood was allowed to flow freely and the first 1 or 2 drops were discarded. This was done under light ether anesthesia as were the injections of the germanium dioxide solution.

The test rats of the first group, both males and females, were injected with 1.2, 1.5, 1.8, and 2.1 mg. of germanium dioxide per kilo of body weight in sterile 0.4 per cent solution on the 8th, 11th, 15th, and 19th days of observation respectively. The solution was made at first slightly alkaline in order to facilitate the dissolving of the oxide. It was then brought back to neutrality by dilute hydrochloric acid. The rats of the second group were injected with ten times the amount given to the first group, on the 8th, 11th, and 15th days of observation. No injections were made on the 19th day because an increased coagulability of the blood had appeared which made difficult the taking of a proper sample. A total of 6.6 mg. per kilo of body weight was injected into the rats of the first group and of 45 mg. into the test rats of the second group.

Two more counts were made of the corpuscles, one on the 22nd and one on the 28th day of the experiment, the last count being made 11 days after the last injection in the first group and after an interval of 14 days in the second group. At this time the experiment was discontinued because the results were adequate, as shown by Tables I to V. The animals were anesthetized with ether and killed by crushing the spinal cord in the cervical region. All values represent the average of two counts made in two counting chambers. In Table V will be found the statistical data concerned with this study. The accepted criterion for observed differences to be statistically valid is that the probable error of the means must be contained at least twice and should be contained three times, for incontestable figures, in the difference between the means. The differences in the erythrocytes between the fore and after periods of the test rats and the differences between the control rats and the test rats are seen to be statistically valid.

The observations conclusively demonstrate that germanium dioxide causes a very marked increase in the number of red cells per cubic millimeter of blood. An increase in the erthyrocyte count of from 1

TABLE I.

The Influence of Small Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Male Albino Rat.

Day.	Control rat.		Test rats.				GeO_2 injected per kilo of body weight.
			No. 1.		No. 2.		
	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
							mg.
1	7.45	7.2	8.17	9.6	7.61	8.3	
7	7.76	6.6	8.39	7.7	7.96	9.3	1.2*
10	—	—	—	—	—	—	1.5
14	7.57	6.5	9.26	8.6	8.42	7.9	1.8
18	—	—	—	—	—	—	2.1
21	7.78	7.1	9.60	7.8	10.37	8.6	
28	7.68	7.8	9.21	9.5	10.34	9.3	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

In Tables I to IV all values for the red cells are in terms of millions, all values for the white cells in terms of thousands.

TABLE II.

The Influence of Small Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Female Albino Rat.

Day.	Control rat.		Test rats.						GeO_2 injected per kilo of body weight.
			No. 1.		No. 2.		No. 3.		
	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
									mg
1	7.73	7.3	7.81	7.3	7.65	8.2	7.67	6.7	
7	7.98	7.7	7.79	7.2	7.86	8.4	7.74	7.4	1.2*
10	—	—	—	—	—	—	—	—	1.5
14	7.90	—	9.38	6.6	9.25	8.6	9.10	8.3	1.8
18	—	—	—	—	—	—	—	—	2.1
21	Killed by acci-		9.68	8.7	10.09	11.3	9.60	7.5	
28	dent.		9.34	7.5	9.58	10.3	8.92	8.6	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

TABLE III.

The Influence of Large Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Male Albino Rat.

Day.	Control rat.		Test rats.						GeO_2 injected per kilo of body weight.
	R. B. C.	W. B. C.	No. 1.		No. 2.		No. 3.		
			R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
									mg.
1	8.64	9.6	9.31	10.2	8.76	10.1	9.38	10.2	
7	8.61	10.4	9.30	12.2	8.70	10.1	9.38	10.1	12.0*
10	—	—	—	—	—	—	—	—	15.0
14	8.72	10.6	11.04	16.8	10.20	12.5	9.82	11.0	18.0
18	—	—	—	—	—	—	—	—	
21	8.95	9.2	10.96	12.2	10.16	8.6	10.79	9.1	
28	8.92	10.4	10.47	10.2	10.40	9.4	11.58	9.5	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

TABLE IV.

The Influence of Large Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Female Albino Rat.

Day.	Control rat.		Test rats.						GeO_2 injected per kilo of body weight.
	R. B. C.	W. B. C.	No. 1.		No. 2.		No. 3.		
			R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
									mg.
1	7.70	13.9	7.38	7.1	9.52	7.0	8.69	8.7	
7	7.81	17.0	7.64	11.8	9.35	10.6	8.60	8.4	12.0*
10	—	—	—	—	—	—	—	—	15.0
14	7.97	20.0	9.20	9.4	10.54	9.1	9.36	9.6	18.0
18	—	—	—	—	—	—	—	—	
21	8.20	18.9	9.40	12.8	11.20	12.4	10.57	9.5	
28	8.01	13.1	12.31	10.3	10.66	10.9	11.56	8.9	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

to nearly 5 million cells occurred in every one of the eleven rats which had had injections of this compound. No such response was exhibited by the control rats. Moreover, germanium dioxide does not produce a leucemia as an accompaniment of the erythrocytosis.

The combination of the fact that germanium dioxide is non-toxic and non-corrosive, with the fact that it produces such a marked increase in the number of the erythrocytes in the circulation of the healthy rat gives us the hope that this compound has a specific stimulating effect upon the erythropoietic tissue and will be found of clinical value.

An inspection of the tables will show that there is a tendency for the red count to increase most in those animals in which the original

TABLE V.

Statistical Data Concerned with the Study of the Influence of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Albino Rat.

	Control rats.				Test rats.			
	Initial counts.		Subsequent counts.		Initial counts.		Counts after GeO_2 injections.	
	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.
Mean.	7,960,000	9,959	8,170,000	11,533	8,395,000	8,931	10,074,000	9,734
Standard deviation.	406,000	3,452	485,000	4,702	713,000	1,547	2,743,000	1,907
Probable error of mean.	97,000	823	104,000	1,057	103,000	222	322,000	223

cell count is lowest. It is also evident that the small doses cause just as marked and significant a rise as do the larger doses.

Attention should be called to the fact that there occurred a slight but statistically valid increase in the erythrocytes of the controls in each series. This increase is attributed to the possibility of food contamination through excretion of germanium, and is supported by the fact that the effect was not observed in rats used as controls in a similar series of studies with arsenic.

At autopsy marked differences were found in the color of the liver and the bone marrow of the germanium-treated rats as compared

with the controls. The liver of the control was a reddish tobacco-brown color, that of the test animals was a reddish purple. The bone marrow of the control albinos was the color of coffee with cream with here and there a slight tinge of red. That of the germanium-injected rats was maroon. No differences in the color of the spleens were detectable. A paper dealing with the histological findings will be published later.[23]

SUMMARY AND CONCLUSIONS.

Injections were made of a sterile 0.4 per cent solution of germanium dioxide into four lots of mature male and female albino rats. Exact conditions of control were maintained. To two lots there was administered in four doses at intervals of 4 days a total of 6.6 mg. of the oxide per kilo of body weight. To two lots there was given in three doses at like intervals a total of 45 mg. of the compound per kilo of body weight. In each lot there was one rat which served as a control and which did not receive any germanium. A preliminary period of observation was maintained as a further control during which two determinations of the erythrocyte and leucocyte counts of the blood were made 7 days apart on all the rats. Weekly counts were made on all the rats during the progress of the investigation which lasted for 4 weeks, during 3 of which the treated animals were under the influence of germanium. In the first group 11 days elapsed between the last injection and the last count. In the second group the interval was 14 days.

It was found that without exception all of the test rats responded to the germanium dioxide by a marked and sustained rise in the number of erythrocytes in the blood which ranged from 1 to nearly 5 millions.

These results are statistically valid.

There was an apparent tendency for the degree of effect to be related to the initial erythrocyte number, in that with a lower initial count there seemed to take place a greater rise, and *vice versa*.

There was no indication that the larger doses of germanium dioxide exerted a greater stimulating effect on the production of the resultant erythrocythemia than the smaller doses.

[23] Hammett, F. S., and Nowrey, J. E., Jr., *J. Exp. Med.*, 1922, xxxv (in press).

There is evidence that the effect is quick in making its appearance. The rise in the red cell count was found to occur within a week, and after but two injections of the oxide.

Indications were obtained that germanium dioxide tends to increase the coagulability of the blood.

Autopsy findings showed color changes in the liver and bone marrow.

We believe that germanium dioxide is an erythrocytogenic agent and we hope that fruitful results will come from its therapeutic application.

THE USE OF PHENOL RED AND BROM-CRESOL PURPLE AS INDICATORS IN THE BACTERIOLOGICAL EXAMINATION OF STOOLS.

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There have been so many new media proposed during the past few years for the isolation from stools of organisms belonging to the typhoid-dysentery group that one hesitates to bring forward a new one. Believing that the indicators phenol red and brom-cresol purple possess distinct advantages over those formerly used in stool bacteriology, we have felt justified in pointing out their applicability in this particular domain of bacteriological technique.

A critical analysis of all the media advocated for the culture of stools is considered beyond the scope of this paper, but a brief mention of some of those more widely used does not seem amiss. In this country Endo's medium, prepared according to the author's formula or according to the modifications advocated by Kendall (1) or Robinson and Rettger (2), has been used perhaps more extensively than any other when a non-restraining medium was desired, although this medium has disadvantages which are well known to all workers in this field. The methylene blue-eosin agar of Holt-Harris and Teague (3) has found favor with some workers because it avoided some of the faults of the Endo medium. Meyer and Stickel (4) consider that either the medium of Holt-Harris and Teague or their own eosin-China blue medium prepared with peptic or tryptic digests is superior to the Endo agar, litmus agar, or Congo red agar "for the direct isolation and detection of dysentery bacilli from stool specimens." The extensive work of Krumwiede (5) and his coworkers upon the usefulness of brilliant green agar as a selective restraining medium has shown that this medium can be successfully applied in routine work when dealing with members of the typhoid-paratyphoid group. Teague and Clurman (6) advocate the use of a meat extract agar containing brilliant green and eosin as a selective medium for the isolation of members of the typhoid-paratyphoid group, and Meyer and Stickel[1] state that eosin and

[1] Meyer and Stickel (4), p. 67.)

brilliant green used in connection with their peptic digest agar "permits the detection of a higher percentage of viable typhoid bacilli than any other one thus far introduced into bacteriologic technic." Kligler (7) finds that the Endo medium after the addition of sulfite reaches a hydrogen ion concentration ranging from pH 8.6 to 8.8 and states that this degree of alkalinity is unfavorable to the growth of the Shiga bacillus. He also advocates the use of neutral red with the brilliant green plate of Krumwiede and considers this the best among several indicators that he tried. Kligler thinks also that the reaction of the medium has a marked influence upon the restraining activity of the brilliant green. It may be mentioned at this point that Krumwiede (8) objects to the use of eosin in connection with brilliant green because of the resultant eye strain from the irritating color produced by this combination. This objection would seem to hold good only where large numbers of plates are to be examined by one worker. Morishima (9) has recently advocated the use of a combination of phenol red and China blue as an indicator for lactose plates in the culture of stools.

In this communication it is proposed to record our experience with the use of phenolsulfonephthalein (phenol red) and dibromo-ortho-cresol-sulfonephthalein (brom-cresol purple) as indicators in stool bacteriology. To Clark and Lubs (10), largely, belongs the credit for the introduction of these indicators into bacteriological technique. They suggest that these indicators might be serviceable in the preparation of indicator plates (11), but so far as our survey of the literature has gone we have not been able to find any reference to the use of these indicators in stool bacteriology except in the case of the combination of phenol red with China blue, as advocated by Morishima.

Method.

In studying these indicators to determine their availability for stool work the basis of the medium used was a 1.5 and a 3 per cent beef extract agar. To this agar were added varying amounts of lactose from sterile solution and varying amounts of indicator, in order to determine the optimum amount of each of these ingredients. The reaction of the medium also was varied in order to ascertain the pH at which sharpest color differentiation was had. Plates were seeded with mixtures of *Bacillus coli* and members of the typhoid-dysentery group, also with stools inoculated with members of the latter. When the optimum amounts of the constituents had been ascertained, brilliant green was added to the agar in the proportions

recommended by Krumwiede, and the degree of restraining activity noted as compared with controls consisting of agar with brilliant green alone and agar with the indicator alone.

RESULTS.

In the case of both indicators a 3 per cent beef extract agar cleared with egg to which 1 per cent lactose had been added from sterile 20 per cent solution gave the best results With the thicker agar there was less diffusion of acid. Thick plates, about 20 cc. of agar to a plate, were poured. We shall discuss the two indicators separately.

Phenol Red.—The optimum reaction of the agar, if this indicator is to be used, is a hydrogen ion concentration represented by a pH of 7.6 to 7.8. The optimum amount of indicator is 10 cc. of a 0.04 per cent aqueous solution for every 100 cc. of agar. The indicator may be sterilized by autoclaving. Plates poured from agar with a pH of 7.6 to 7.8 and containing the amount of indicator mentioned will assume on cooling a color best described as ranging between salmon pink and old rose. Such plates are clear, and upon them lactose-fermenting organisms produce vivid greenish yellow colonies with a surrounding zone of green. The typhoid bacillus, the paratyphoid bacillus (A and B), and the dysentery bacilli (Shiga, Flexner, and Hiss Y), all produce pink colonies. These organisms evidently produce alkali, for the surrounding medium assumes a decidedly deeper pink than the uninoculated portions of the plate. This alkali production with consequent intensification of the pink color of the medium in the neighborhood of the typhoid-dysentery colonies is a helpful factor in the differentiation of the latter. In thickly seeded plates the diffusion of the acid in the vicinity of the lactose-fermenting colonies is often sufficient to mask this change in the direction of alkalinity exhibited by the typhoid-dysentery colonies, but where good distribution is obtained the color change is most striking. Even in thickly seeded plates where typhoid and colon colonies abut upon one another, a difference in the color of the two types of colonies is observable. The colon colonies assume a bright green or yellow-green color and are opaque, whereas the typhoid colonies in such cases are more translucent and possess a bluish green color. The dye does not mask the corrugated, woolly appearance of the typhoid

colonies which is characteristic of them and upon which Krumwiede lays great stress in their recognition. On the contrary, we are inclined to feel that the dye perhaps enhances this characteristic. We have had no opportunity to study freshly isolated strains belonging to the dysentery group, and it is possible that such strains may be inhibited by this indicator; however, it does not inhibit the growth of freshly isolated strains of *Bacillus typhosus* or *paratyphosus*. The indicator itself is not reduced by the bacteria. We have found it very satisfactory when used in connection with sugar broth or Russell's double sugar.

Brom-Cresol Purple.—In our experience this indicator, when used in connection with lactose plates, gives a sharper color differentiation than does phenol red and hence is preferable to the latter. It is purple in alkaline solution, changing to yellow in the presence of acid. The color change takes place in the zone of pH 5.2 to 6.8. The optimum reaction of the agar if this indicator is to be employed is a pH of 7.2 to 7.4. The optimum amount of indicator to use is 5 to 8 cc. of a 0.04 per cent aqueous solution for every 100 cc. of agar. This solution may be sterilized in the autoclave. Plates poured from agar with a reaction of pH 7.2 to 7.4 and with the amount of indicator mentioned assume a deep blue color and are clear. Upon these plates the lactose-fermenting organisms produce greenish yellow colonies with a yellow zone, and the non-lactose fermenters produce bluish colonies. The typical appearance of the typhoid colonies is not obscured by this indicator. Even on thickly seeded plates the colonies of the non-lactose-fermenting organisms tend to preserve a bluish appearance, being bluish green rather than a brilliant yellow, the color that the colon bacillus colonies assume. It is not an infrequent experience on thickly seeded plates to see a round yellow colon colony with a wedge-shaped bluish green sector occupying a portion of the circle and representing a typhoid colony which has refused to be masked by the larger, acid-producing colony of colon bacilli. This indicator did not show any restraining influence upon any of our laboratory cultures of *Bacillus dysenteriæ*, including the Shiga variety. Whether or not it would exercise any restraint on freshly isolated cultures we are not able to say.

Combination of Phenol Red or Brom-Cresol Purple with Brilliant Green.—To judge from our rather limited experience either indicator

may be used along with brilliant green in the amounts advocated by Krumwiede. We have had an opportunity to study only one sample of brilliant green, the source of which was unknown but which was active in its restraining power upon *Bacillus coli*. Neither indicator, so far as could be determined, exercised any inhibiting effect upon this restraining power of the brilliant green. The only change noticed was in the color of the plates, the phenol red-brilliant green plates being brownish red in color and the brom-cresol purple-brilliant green plates being bluish purple. The characteristic color changes produced by the lactose-fermenting colonies were not altered by the brilliant green.

DISCUSSION.

Since the introduction of lactose indicator plates for the bacteriological examination of stools for the detection of members of the typhoid-dysentery group, numerous attempts have been made to find a medium which was entirely satisfactory. As pointed out by Hiss and Zinsser (12) the best results are apt to be attained when an individual becomes thoroughly familiar with one type of medium and restricts himself to it. Endo's medium has the disadvantage of tending to vary with different batches, some being satisfactory and others useless. There can be little question but that the selective restraining media have been highly successful in skilled hands when large numbers of stools have had to be examined. Few workers, however, in carrying out routine examinations with such media, are willing entirely to dispense with a non-restraining medium such as the Endo plate, but prefer to use it in addition as a control. This is particularly apt to be the case in laboratories serving hospitals, where the proportion of stool cultures to other bacteriological examinations is not great and where the workers cannot be expected to attain the degree of skill in stool bacteriology which is attained by those carrying out an extensive series of bacteriological examinations of stools. For the less experienced worker, let us say, a non-restraining medium is essential, if only as a check, and a medium which does not obscure the characteristic appearance of the typhoid colonies and at the same time shows a sharp color differentiation should be satisfactory for the purpose.

It is because we believe that the indicators phenol red and brom-cresol purple may be successfully employed with lactose to give a

satisfactory differential plate for members of the typhoid-dysentery group when a non-restraining medium is desired and that such a medium is superior to the customary Endo plate that we have ventured to add another medium to the already long list of media proposed for the isolation of typhoid and dysentery bacilli from stools. We think the medium proposed is superior to Endo's because it gives sharper differentiation than the latter, is easier to prepare, uniform batches can be obtained, and it can be kept indefinitely if not allowed to evaporate. Of the two indicators, we prefer brom-cresol purple to phenol red.

CONCLUSIONS.

1. Either phenol red or brom-cresol purple may be used as indicators in the preparation of lactose agar plates for the isolation of members of the typhoid-dysentery group of bacteria from stools. Of the two, brom-cresol purple gives sharper differentiation and is to be preferred.

2. These indicators exercise no restraining influence upon the growth of cultures of the typhoid bacillus or paratyphoid bacillus freshly isolated from the human body, or of laboratory cultures of *Bacillus dysenteriæ*.

3. Both indicators may be successfully employed with brilliant green in the isolation of the typhoid-paratyphoid group from stools without sacrificing the restraining activity of the brilliant green upon other bacteria.

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ROENTGEN RAY INTOXICATION.

I. Unit Dose over Thorax Negative—over Abdomen Lethal. Epithelium of Small Intestine Sensitive to X-Rays.

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The papers of this series form a part of the general program of study of the "non-specific intoxications" which is being carried forward in this laboratory. They are concerned with an investigation of the systemic intoxication which develops after a suitable exposure to large doses of hard Roentgen rays. These papers follow along the logical development of this study, based on the beginnings made by Hall and Whipple (3). Reference to the paper of Hall and Whipple is necessary for a review of some of the rather extensive literature of this subject and for a discussion of many of the fundamental problems related to Roentgen ray intoxication.

This Roentgen ray intoxication, we believe, is a true "non-specific intoxication" and deserves thorough study in part for this very reason. Too little study has been directed toward an understanding of the "non-specific intoxication" which often is a very important factor in many of the so called specific infections. This Roentgen ray intoxication closely resembles the intoxication of intestinal obstruction, another important "non-specific intoxication." We shall point out some important differences between the intoxication due to the Roentgen rays as contrasted with intestinal obstruction, but there remain many striking similarities as emphasized by Hall and Whipple (3).

We believe our experiments make it clear that the fundamental thing in the systemic intoxication due to the Roentgen rays is a *primary injury of the epithelium of the small intestine.* This was sus-

pected by Hall and Whipple but not conclusively proven. Our experiments in normal dogs under controlled conditions appear convincing when exposures given over the thorax are contrasted with the same exposures over the abdomen. For example, a unit dose (350 milliampere minutes) given over the thorax (abdomen shielded) causes no clinical disturbance in the dog. A subsequent exposure to the same unit dose in the same dog given over the abdomen (thorax shielded) will cause fatal intoxication and death in 4 days. For a normal 30 to 40 pound dog, we may say that 350 milliampere minutes given over the abdomen is a minimum lethal dose.

The outstanding abnormalities in the abdominal exposures are the remarkable necroses observed in the small intestine. Large areas may show complete necrosis and disappearance of the intestinal epithelium covering the villi and lining the crypts. The villi are left as naked polyps made up of stroma, vessels, and a few wandering cells. The inflammatory reaction is not as intense as would be expected under such conditions with complete removal of the covering epithelium. This important point will be discussed in detail in a subsequent paper.

Regaud, Nogier, and Lacassagne (5) described the chronic gastrointestinal lesions in dogs and noted especially gastric atrophy and intestinal perforation. Fromme (2) noted especially the clinical symptoms of gastrointestinal disturbance in guinea pigs and mice. Denis, Martin, and Aldrich (1) noted in rabbits the toxic effect of abdominal radiation and certain abnormalities in the intestines which they state are secondary to an unknown toxic factor.

Method.

The procedure is identical in all experiments, unless otherwise indicated. Normal dogs are used throughout and are kept in standard metabolism cages which have sharply pitched floors to facilitate the collection of urine. To insure the acidity of the cage collections, about 1 cc. of glacial acetic acid is added to the cage collecting bottle at the beginning of each 24 hour collection. The dogs are catheterized daily at 10 o'clock. The cage collections, cage washings, bladder washings, and catheterized urine are mixed and diluted to a unit volume—usually 2 liters. The total nitrogen is then done in dupli-

cate by the Kjeldahl method. About 300 cc. of warm water is given daily by stomach tube as a routine procedure after the dog is catheterized. Water is available at all times in the cage.

Dogs are given a subcutaneous injection of morphine shortly before exposure to radiation. A medium hard Coolidge tube is used with the target (anode or anticathode) set at a constant distance of 10 inches from the skin. At this distance the effective radiation for this machine covers a circle approximately $6\frac{1}{2}$ inches in diameter. The current strength used is either 7.5 or 8.0 milliamperes. The E.M.F. varies considerably in different experiments from 85 kilovolts to 103 kilovolts. The spark-gap backing up these voltages varies also, measuring from $8\frac{1}{2}$ to 10 inches between sharp points. The amount of radiation is calculated as the product of the current strength in milliamperes and the time of exposure in minutes and is expressed as milliampere minutes. In all these experiments the standard autotransformer x-ray equipment of the University of California Hospital is used. Aluminum filters (2 mm.) are used to cut out the softer rays. The various parts of the dog were effectively protected from radiation, when it was so desired, by sheets of lead-foil approximately 2 mm. thick, or by lead "rubber," approximately 5 mm. in thickness.

The body of the dog is marked off roughly into two parts by a transverse line at the level of the xiphisternum. The part between this line and the base of the neck is designated as the thorax, that below as the abdomen. Each of these parts is then divided into an upper and lower half. Each upper and lower segment of the abdomen or thorax is radiated from the lateral and midventral aspects. Thus the ventral portion of the abdomen or thorax is exposed to radiation over six different areas whose contiguous edges overlap somewhat. Usually one exposure is given over the middorsal region of the thorax or abdomen, causing the total dosage to be divided up into seven parts, which, however, are spread rather diffusely over the part of the body involved. All dogs are sacrificed under chloroform or ether anesthesia by bleeding.

EXPERIMENTAL OBSERVATIONS.

Some of our typical experiments are tabulated below and make several points quite clear. However, we wish to refer to other experiments of similar nature given in subsequent papers of this series. These experiments are all in agreement and indicate that enormous doses of Roentgen rays may be given over the thorax without causing any clinical reaction in a normal dog. Dogs have been given amounts of radiation varying from 300 to 512 milliampere minutes over the thorax without any clinical symptoms. One dog (No. 19–117) was given 350 milliampere minutes over the thorax on three separate occasions with intervening periods of 2 to 3 weeks; all this without any apparent cumulative effects or clinical reaction. There is usually a slight transient leucopenia and there may be a very slight rise in total nitrogen excretion. After 2 or 3 weeks, the skin shows loss of hair and pigmentation as a result of the exposures. The ribs of the animal examined at autopsy show a considerable loss of marrow cell elements. We have been able to recognize no other results from these large doses of x-rays over the thorax.

Dog 19–78 (Table I) is a typical example of a complete experiment to show that a unit dose of x-rays is clinically inert over the thorax but lethal when given over the abdomen. The thorax dose causes a definite leucopenia but no clinical disturbance. That the second dose does not cause as much of a leucopenia, we believe, may be due to the tissue injury in the small intestine.

The radiation over the abdomen causes a typical clinical reaction and the review of this case will suffice for all subsequent experiments. This clinical reaction is remarkably constant and is described in detail below (Dog 19–78). The latent period of 24 to 36 hours is noted as usual and will be discussed later. The rise in total urinary nitrogen is not great but is significant. The figure for the last day includes a certain amount of fecal contamination.

Dog 19–78. Clinical History and Autopsy (See Table I).

Jan. 27. The dog is isolated on fasting diet. The animal is in good condition and quite active.

Jan. 29. The abdomen is covered with lead-foil well up to the level of the diaphragm. The thorax is exposed in seven areas, two midventral, two on each

side, and one over the midscapular region. Each area exposed covered a patch of skin approximately 5 inches square. Morphia ($\frac{5}{8}$ gr.) is given to keep the dog quiet during the exposure. The current strength is 7.5 milliamperes at an E. M. F. of 100 kilovolts with the spark-gap set at 9 $\frac{9}{16}$ inches. 2 mm. of aluminum filter is used and the tube is set at a target-skin distance of 10 inches.

TABLE I.

Roentgen Radiation.

Unit Dose Given over Thorax and Subsequently over Abdomen.

Dog 19–78. Adult, female.

Date.	Weight.	White blood corpuscles per c. mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	lbs.		*cc.*	*gm.*		
Jan. 28	26.0	—	—	—	Water.	Good condition.
" 29	26.0	7,600	—	—	"	
Jan. 29	X-rays, 350 milliampere minutes, given over thorax. Abdomen shielded.					
Jan. 30	25.8	8,000	—	—	Water.	Dog normal.
" 31	24.5	4,100	—	—	"	
Feb. 1	23.8	4,600	—	—	"	Dog normal.
" 2	23.3	4,600	—	—	"	
" 3	23.0	—	—	—	Mixed.	Dog normal.
" 8	27.0	5,400	—	—	"	
" 21	28.3	7,400	—	—	"	Dog normal.
	Experiment ended.					
Mar. 15	28.9	7,800	—	—	Water.	Dog normal.
" 17	27.8	7,600	260	2.74	"	
" 18	27.3	8,000	305	2.68	"	Dog normal.
Mar. 18	X-rays, 350 milliampere minutes, given over abdomen. Thorax shielded.					
Mar. 19	26.8	6,400	290	2.83	Water.	Dog normal.
" 20	26.6	7,000	265	2.74	"	Inactive.
" 21	25.9	7,200	240	3.00	"	Vomitus and diarrhea.
" 22	25.0	3,900	—	5.63	"	Moribund.
	Chloroform anesthesia and autopsy at once.					

Jan. 30. Dog remains normal during entire experiment (see Table I). There was a slight transient leucopenia. Dog was on mixed diet until the second experiment, also given in Table I.

Mar. 15. The dog is active and in good condition.

Mar. 18. Dog given ½ gr. of morphia 1 hour before exposure to radiation. The thorax and head and neck are thoroughly screened with lead-foil down to the tip of the xiphisternum. Seven areas are exposed, two midventral, two on each lateral surface, and one midlumbar. The current strength is 7.5 milliamperes at an E. M. F. of 95 kilovolts, backed by a 9½ inch spark-gap. The radiation is filtered by 2 mm. of aluminum. The skin-target distance is 10 inches. There are no immediate after effects from exposure to 350 milliampere minutes.

Mar. 19. The dog is bright and active.

Mar. 20. Slight evidences of intoxication are manifested by the dog—one soft, yellow, watery stool.

Mar. 21. The intoxication becomes marked in the afternoon. The diarrhea has progressed from brownish, watery material to tarry, black mucous or bloody mucous material with a very foul odor. Vomitus increases in amount and frequency during the day.

Mar. 22. The animal is very weak and dull. In the afternoon, the dog is completely prostrated. The stools have become very frequent and of large volume. The pulse is irregular and thready. The temperature has risen to 40.2°C. The respirations are rapid and irregular. The animal is practically moribund and is sacrificed under chloroform anesthesia.

Autopsy.—Performed immediately after death by chloroform. The hair over the thorax is thinned. It is absent over the sternum where the skin is somewhat pigmented from previous exposures to radiation. Pleural cavity, heart, and lungs are normal. Peritoneal surfaces are normal. Spleen is somewhat granular in appearance, hard and firm, normal in size. On section, the trabeculæ stand out. Mesenteric glands slightly enlarged and moist—somewhat pinkish in color. Kidneys normal in size and appearance. Bladder contracted; it shows a few ecchymoses in its mucosa (catheter). Liver is normal. Adrenals are somewhat enlarged; apparently normal except for a small necrotic mass in the left adrenal.

Stomach contains gas and about 20 cc. of a bile-stained fluid. The mucosa is pale and normal.

Duodenum is somewhat thick-walled and spastic. The lumen contains many large, round worms and a small amount of reddish-black mucus. Beginning sharply at a point approximately an inch distal to the *pyloric valve*, the mucosa of the *duodenum* presents a dark red glassy appearance. On this background run several longitudinal folds with bright red hemorrhagic crests. Numbers of small bright red petechiæ show up between these plicæ on the dark, glassy surface.

The reaction in the *jejunum* seems less intense, for here the mucosa is paler and the plicæ are not so conspicuous though they can be distinctly made out.

Ileum contains a small amount of reddish-black mucous material similar to the stools, and a few round worms. The intestinal wall is thin and rather flaccid and on close inspection the intensely hemorrhagic mucosa surface seems to have been denuded of most of the villi as the velvety appearance is lacking. The longitudinal folds with bright red hemorrhagic crests are conspicuous. Peyer's

patches seem to be stripped of their villous covering so that they show up readily as speckled oval patches. The ileocecal valve marks an abrupt end of this extensive injury.

Colon mucosa is pale, though a few longitudinal, hemorrhagic folds are present. The walls are spastic and the lumen contains a small amount of reddish fluid.

Histological Sections.

Lungs, heart, liver, pancreas, kidneys, bladder, uterus, and stomach are negative. The mesenteric lymph nodes are slightly hemorrhagic and contain some pigment. The spleen is moderately atrophic and contains pigment. In the ovary the ova are apparently dead, though nucleoli and chromatin remnants are still present within the nuclear membrane.

Pyloric duodenum: a section shows the transition from normal intact pyloric villi to collapsed and denuded villi with remnants of dead epithelium and some regenerating epithelial cells undergoing mitosis. Some areas have escaped injury.

Jejunum: destruction and disappearance of the epithelium of villi and crypts; a few crypts remain, otherwise the normal structure is gone. The epithelium around these crypts shows mitotic figures and these are apparently regeneration forms. Large pale epithelial cells stick out into the lumen of the crypts. Their cytoplasm is finely meshed and is decidedly eosinophilic. The nuclei are rather large and pale and frequently contain mitotic figures. A large amount of hemorrhage has occurred in the submucosa, and blood vessels are occasionally found plugged with thrombi of fibrin and platelets. On some areas a very thin sheet of epithelial cells can be seen making an attempt to recover the collapsed villi.

The *ileum* shows extreme injury, with the disappearance of the greater part of the crypt and villous epithelium. Strenuous attempts at regeneration are being made by remnants of the epithelium which shows many mitotic figures and is found clumped in little groups or sheets as if these groups were descendants of one or two surviving cells of the original epithelium.

The *colon* shows some mucoid degeneration and slight necrosis of the tips of a few villi, with evidence of repair.

Dog 19–85. Clinical History and Autopsy (See Tables II and IV).

Dog 19–85 received 300 milliampere minutes over the abdomen (Table IV) which was followed by diarrhea and vomitus and prostration. She recovered. 1 month later she was given 350 milliampere minutes over the thorax with no effect (Table II). A month later (2 months after the first exposure) she was given 350 milliampere minutes over the abdomen with fatal result (Table II).

Mar. 2. Dog fasting since Feb. 28.

Mar. 4. Dog given ¼ gr. of morphia 1 hour before exposure to radiation. Lower part of body screened up to xiphisternum by lead-foil 2 mm. thick; 350 milliampere minutes of radiation divided up into the usual seven areas. A cur-

rent strength of 7.5 milliamperes is used at 103 kilovolts and backed up by a 9⅞ inch spark-gap. A 2 mm. aluminum filter is used. Skin-target distance 10 inches. The total time of exposure is 46.6 minutes. There are no apparent after effects from the x-ray exposure.

Mar. 5 to 8. The dog remains clinically normal.

TABLE II.

Roentgen Radiation.

Unit Dose over Thorax Negative but Subsequent Unit Dose over Abdomen Lethal.

Dog 19–85. Adult, female.

Date.	Weight.	White blood corpuscles per c.mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	lbs.		*cc.*	*gm.*		
Mar. 2	24.7	—	—	—	Water.	Good condition.
" 3	24.4	7,800	355	2.04	"	
" 4	24.1	6,800	310	1.71		Dog normal.
Mar. 4	X-rays, 350 milliampere minutes, given over thorax. Abdomen shielded.					
Mar. 5	23.8	6,600	290	1.90	Water.	Dog normal.
" 6	23.4	8,000	325	1.79	"	" "
" 7	23.2	5,400	325	1.96	"	" "
" 8	22.9	6,800	—	—	"	
" 9	22.8	—	—	—	Mixed.	
	Experiment ended.					
Mar. 30	25.0	—	—	—	Water.	Dog normal.
" 31	24.6	—	440	2.07	"	
Apr. 1	24.1	7,400	325	1.90	"	Dog normal.
Apr. 1	X-rays, 350 milliampere minutes, given over abdomen. Thorax shielded.					
Apr. 2	23.4	7,300	300	2.13	Water.	Dog normal.
" 3	23.1	5,800	290	1.90	"	" "
" 4	22.8	7,200	240	2.07	"	Diarrhea and vomitus.
" 5	22.1	5,400	275	3.16	"	Severe intoxication.
	Ether anesthesia and autopsy at once.					

Mar. 30. Dog has remained normal since the exposure over the thorax. There is some depilitation and pigmentation of previously exposed areas. Dog fasting for past 3 days.

Apr. 1. Animal given ⅜ gr. of morphine 1 hour before exposure; 350 milliampere minutes of radiation spread diffusely over the abdomen in six areas, all ventral. Each area averages approximately 25 square inches. The current strength

is 7.5 milliamperes at an E. M. F. of 90 kilovolts, backed up by a 9 inch spark-gap. A 2 mm. aluminum filter is used. Skin-target distance 10 inches. There are no clinical after effects which could be attributed to the radiation.

Apr. 2 and 3. The animal is active and lively.

Apr. 4. There is quite a large volume of yellow, semifluid feces and some bile-stained vomitus throughout the morning. Intoxication becomes more marked in the afternoon. The pulse is regular, but of low tension.

Apr. 5. Greenish-brown, semifluid stools and bile-stained vomitus appear regularly throughout the day. In the afternoon, the stools are tarry and vile smelling. In general, the stools appear to contain digested or partly digested red blood cells, mixed with mucus. The vomitus at no time contains any blood, but is made up of a rather opalescent fluid containing thick, stringy mucus, which at times is bile-stained. The animal is markedly intoxicated, especially during the afternoon. Dog is able to walk but quite weak and ceases to respond voluntarily. The animal is not moribund and might survive, but in all probability would die during the night. Ether anesthesia and autopsy at once.

Autopsy.—Body fat well preserved. Pleural cavity and thoracic viscera are negative. Peritoneal cavity contains no fluid and the peritoneal surfaces are normal. Liver, spleen, pancreas, adrenals, kidneys, and genitourinary tract are negative.

Stomach contains about 20 cc. of bile-stained, frothy mucus, corresponding to the vomitus of the past 2 days. The mucosa is pale and apparently negative.

Small intestine is somewhat spastic and contains a small amount of greenish-brown mucous material.

Longitudinal folds with hemorrhagic crests are conspicuous in the duodenum. These fade out and disappear in the upper jejunum. In both the duodenum and ileum, there are patches or clusters and streaks of tiny ecchymotic specks in the mucosa. The superficial necrosis is not very extensive but stands out in contrast to the normal patches of mucosa in the jejunum and upper ileum, which are pale and velvety and apparently undisturbed.

Colon is negative.

Histological Sections.

Lungs, liver, pancreas, kidneys, adrenals, bladder, and uterus are negative. Ovary: the ova are dead, the nuclear material is broken up, and the cytoplasm is foamy. Spleen: atrophy of the Malpighian bodies and pulp elements; a few fragmented nuclei are seen. Stomach: normal. Duodenum: irregular and patchy injury of villus structures is apparent. In injured areas, there are evidences of mitosis and rapid growth of epithelial cells and some infiltration of the area by polymorphonuclear leucocytes. The irregularity of the injury is especially noted in the sections from the jejunum.

Ileum shows extensive injury and destruction of villous epithelium. The epithelium covering the villi and crypts has disappeared except for some rapidly

growing epithelial cells apparently regenerating in an effort to cover the denuded areas. Polymorphonuclear and mononuclear leucocytes are rather numerous at the bases of the crypts.

Colon shows a certain amount of mucoid degeneration and here and there an occasional focus of polymorphonuclear infiltration especially under the areas where the epithelium is injured.

Dog 19–101. Clinical History and Autopsy (See Table III).

Feb. 17. Dog is active and in good condition.

Feb. 21. ½ gr. of morphia is given 1 hour previous to the radiation; 117 milliampere minutes of radiation are given with a current strength of 7.5 milliamperes,

TABLE III.

Roentgen Radiation.

350 Milliampere Minutes over Thorax Negative.

Dog 19–101.

Date.	Weight.	White blood corpuscles per c. mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	lbs.		*cc.*	*gm.*		
Feb. 17	34.0	8,000	—	—	Water.	Dog normal.
" 20	31.0	—	—	—		
" 21	30.4	7,800	400	3.03		
Feb. 21	X-rays, 350 milliampere minutes, over thorax. Abdomen shielded.					
Feb. 22	30.1	7,300	415	3.02	Water.	Dog normal.
" 23	30.0	7,900	605	2.86	"	
" 24	29.1	5,600	565	2.69	"	
" 25	28.8	7,300	605	2.52	"	
" 26	28.1	7,600	408	2.49	"	
" 27	28.1	7,400	370	2.24	"	Dog normal.
" 28	27.8	6,900	335	2.21	"	
	Experiment ended.					
Apr. 11	31.3	7,600	—	—		
	Ether anesthesia and autopsy at once.					

at an E. M. F. of 103 kilovolts. It was then found necessary to give the remaining 233 milliampere minutes at a current strength of 10 milliamperes with an E. M. F. of 90 kilovolts. 2 mm. of aluminum filter used, spark-gap of 9 inches between sharp points. The skin-target distance is 10 inches.

Apr. 7. Dog is exposed to 450 milliampere minutes of radiation over the abdomen and sacrificed under ether anesthesia on Apr. 11.

Autopsy.—The pleural and pericardial surfaces are moist and shiny. The heart and lungs are negative. The other changes do not concern the experiment given in Table III.

Dog 19–50. Clinical History and Autopsy.

To be contrasted with Table III; a control experiment over the abdomen.

Feb. 10. Dog active and normal; fasting.

Feb. 11. ½ gr. of morphia 1 hour previous to radiation; 340 milliampere minutes are spread over the abdomen in the usual seven areas; screening of the thorax and lower pelvis with lead-foil. A current strength of 7.5 milliamperes at 97 kilovolts is backed up by a 9½ inch spark-gap; 2 mm. of aluminum filter the radiation. The skin-target distance is 10 inches.

Feb. 12. The dog is lively and active. A small amount of white frothy vomitus and some fluid brown feces appear on this day.

Feb. 13. There is evidence of slight intoxication. The pulse tension is low. The vomitus is of large volume, as much as a pint in an hour, and is deeply bile-stained. The diarrhea is frequent but scanty in amount and consists mostly of blood. At times, it is black, thick, and tarry. The dog is quite weak.

Feb. 14. The intoxication is quite severe. The animal is weak. The pulse is of low tension and quite irregular. The vomitus is large in amount and deeply bile-stained. The diarrhea is profuse and frequent. At times, the fecal material seems to be made up practically of pure blood and contains many clots. Again it may be tarry and vile smelling. The dog is comatose and is sacrificed under ether anesthesia. Autopsy at once.

Autopsy.—Thoracic viscera and serous surfaces are negative. The liver, spleen, adrenals, kidneys, lymph nodes, and genitourinary tract are negative. The pancreas shows a few small fat necroses.

Stomach: filled with a bloody mucous material; mucosa pale and normal.

Small intestine is spastic and contains a small amount of bloody fluid. The mucosa is a dull red, darker in the duodenum, lighter in the ileum. Longitudinal folds with hemorrhagic crests are evident in some sections, especially the paler sections of the lower jejunum, but they are masked in the duodenum by the intense dark dull red of the mucosa there. The intestinal wall is thin, apparently due to much loss of substance in the mucosa. This is especially marked in the ileum.

Colon and *cecum* contain a bloody fluid. Their mucosa is in general pale.

Histological Sections.

Liver, kidneys, adrenals, uterus are normal. Spleen shows some pulp atrophy and prominence of the reticulum. Pancreas contains a few fat necroses in one section, otherwise it is normal. In the ovary, the chromatin network of the ova is broken up, though the nucleoli are still present. The ova in general appear abnormal and seem to be degenerating.

Stomach and *colon* are normal.

Small intestine presents the usual picture of necrosis and destruction of villous and crypt epithelium, regeneration of epithelium with mitotic figures, and infiltration of polymorphonuclears into the bases of the crypts.

In the *ileum* the villi show only a naked reticulum with a few groups of regenerating epithelial cells and the lymph follicles. The blood vessels in the mucosa are frequently thrombosed.

The *mesenteric lymph nodes* are filled with red blood cells and polymorphonuclears and phagocytic cells containing cell debris and pigment.

TABLE IV.

Roentgen Radiation.

Sublethal Dose over Abdomen.

Dog 19–85.

Date.	Weight.	White blood corpuscles per c. mm.	Diet.	Remarks.
	lbs.			
Feb. 4	25.0	8,000	Water.	Dog normal.
Feb. 4	X-rays, 300 milliampere minutes, over abdomen. Thorax shielded.			
Feb. 5	24.5	6,000	Water.	Dog normal.
" 6	24.0	8,000	"	
" 7	23.5	3,400	"	Inactive.
" 8	23.3	8,400	"	Vomitus and diarrhea.
" 9	23.0	5,800	"	No diarrhea or vomitus.
" 10	23.0	7,800	"	Clinical improvement.
" 11	22.5	5,400	Mixed.	
" 20	25.0	7,400	"	Good condition.

Dog 19–85. Clinical History (See Table IV).

Feb. 3. Dog active and in good condition; fasting.

Feb. 4. ½ gr. of morphia given 1 hour before radiation. The upper part of the body above the level of the xiphisternum is screened by 2 mm. of sheet lead-foil. Exposures made over the abdomen and pelvis in seven parts. The current strength is 7.5 milliamperes at an E. M. F. of 100 kilovolts, backed up by a 9½ inch spark-gap. The radiation is filtered by 2 mm. of aluminum. The skin-target distance is 10 inches. The total exposure time is 40 minutes—300 milliampere minutes. The dog is clinically unaffected by the exposure to radiation.

Feb. 5 to 7. Dog normal.

Feb. 8. The animal shows a slight degree of intoxication. The vomitus which appears is mostly a clear frothy mucus. Diarrhea is brownish and scanty.

Feb. 9. Mild intoxication; no diarrhea or vomitus.

Feb. 10. From this date, recovery to normal is rapid.

DISCUSSION.

At the very start of this discussion, we wish to point out the fact that these experiments concern normal dogs and not human beings suffering from disease, for example leucemia, cancer, and so forth. We feel that these experiments are less complicated for this very reason and may give us the opportunity to correct mistaken impressions gotten from the complex reactions which may result from Roentgen radiation of a diseased human being.

Our negative results of thorax radiation (up to 512 milliampere minutes) are of interest in ruling out convincingly certain of the body cells which have been suspected as causative factors in the general Roentgen ray intoxication. For example, these enormous doses given over the normal thorax must injure more bone marrow cells than do the abdomen exposures. The ribs and thoracic vertebræ surely contain more units of red marrow than do the lumbar vertebræ. Examination of the rib marrow some days after an exposure shows almost complete disappearance of red marrow cells, so we know that this reaction has taken place, yet there is not a trace of any clinical disturbance. This rules out the red marrow as a factor in the characteristic systemic disturbance noted in our experiments.

These same arguments apply to lymphatic tissue. The lymph glands in thorax and abdomen usually show some loss of cells but not to any very great extent. It is significant that Peyer's patches in the intestine may show only moderate injury when the epithelium covering the lymph follicles and lining the crypts has completely vanished due to the x-ray injury. In our experiments, therefore, the intestinal epithelium is much more sensitive to injury than are the cell elements of the lymph gland and Peyer's patch.

The spleen of the dog is not profoundly affected by the Roentgen radiation. For one thing, it is not as cellular as the human spleen, but we feel that the normal spleen cells are not easily destroyed by the x-rays. After large exposures we note a moderate atrophy of the

spleen with shrinkage of the Malpighian corpuscles, but plenty of lymphocytes remain even after intensive radiation.

The thorax exposures, of course, cover the heart and lungs, which may be dismissed without comment. This area also includes a large part of the *circulating blood*. We conclude, therefore, that the x-rays have no injurious effect upon the circulating blood cells.

The thorax exposures therefore rule out several factors which at one time or another have been thought to be responsible for the general systemic intoxication—red bone marrow, lymphatic tissue, and circulating blood. We may now consider the various tissues in the abdomen which may be responsible for the fatal reaction following large doses of the x-rays.

The kidneys are very slightly disturbed, as is shown in a paper of this series (McQuarrie and Whipple (4)).

The liver is not concerned and we believe can be ruled out without question. There is no histological evidence of injury, but more than that, there is no evidence of functional disturbance. Dogs with bile fistulæ show no disturbance in function and no change in the level of bile acid and bile pigment output.[1]

The pancreas and adrenals show no histological evidence of injury by the x-rays. We know of no functional disturbance which may be attributed to these structures.

The gastrointestinal tract is remarkably sensitive to the Roentgen rays. In subsequent experiments, we shall review the remarkable ulcers which can be produced in the stomach, but as a whole, the stomach and colon are considerably more resistant than the small intestine.

The *epithelium of the small intestine* from the pylorus to the ileocecal valve is remarkably sensitive to the Roentgen rays. These doses used will cause necrosis of this epithelium with practically complete disappearance. We believe the symptoms of intoxication and the final lethal poisoning are due to this injury of the intestinal epithelium. We need not refer to the severe intoxication which develops following a functional disturbance of this same epithelium (intestinal obstruction). We may infer that the x-rays may produce sublethal injury

[1] Experiments to be published elsewhere.

to this intestinal epithelium which may be responsible for a variety of disturbances associated with gastrointestinal symptoms.

SUMMARY.

Roentgen radiation of the thorax (abdomen shielded) in dogs, even with large doses (up to 512 milliampere minutes), gives no clinical evidence of intoxication. There may be a transient leucopenia and a slight rise in urinary nitrogen.

Roentgen radiation of the abdomen (thorax shielded) in dogs, with a dose of 350 milliampere minutes, will almost certainly cause a fatal intoxication. Smaller doses may be survived but usually with signs of gastrointestinal intoxication.

This lethal intoxication due to abdominal radiation presents a remarkably uniform clinical and anatomical picture. There is a latent period of 24 to 36 hours, during which the dog is perfectly normal clinically. The 2nd day usually shows the beginning of diarrhea and perhaps some vomitus. The 3rd and 4th days show progressive intoxication with increasing vomiting and bloody diarrhea until the dog becomes stuporous. Death is almost always on the 4th day.

Anatomically the only lesions of significance are to be found in the small intestine. The epithelium of the crypts and villi shows more or less complete necrosis, and this condition may involve almost all of the small intestine. The epithelium may vanish completely except for a few cells here and there which have escaped and are often found in mitosis, probably an effort at repair and regeneration.

We are forced to the conclusion that this remarkable injury of the epithelium of the small intestine is responsible for the various abnormal reactions and final lethal intoxication which follow a unit dose of Roentgen radiation over the abdomen of a normal dog. This sensitiveness of the intestinal epithelium to x-rays is not appreciated and should be given proper consideration in clinical work.

In conclusion we wish to express our sincere appreciation for the assistance and advice given us by members of the Department of Roentgenology of the University of California Hospital—Drs. Howard E. Ruggles, Lloyd Bryan, Orrin S. Cook, and Elizabeth Schulze.

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ROENTGEN RAY INTOXICATION.

II. A Study of the Sequence of Clinical, Anatomical, and Histological Changes Following a Unit Dose of X-Rays.

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The experimental data given below supplement that of the first paper of this series. All these experiments make it very clear that the initial injury of the epithelium of the small intestine is responsible for the severe clinical reaction and lethal intoxication. The fact that this epithelium is sensitive to the Roentgen rays has not hitherto been clearly recognized. For this reason, it seemed desirable to study carefully this reaction and record the histological and gross changes which follow day by day after the initial exposure.

It is well to recall that the urinary nitrogen in these experiments shows a prompt increase on the day following the exposure (experiments in Paper I and those of Hall and Whipple (2)). This increase is not great and no considerable rise is usually noted until the last day before death. This urinary nitrogen curve does not in any way parallel the clinical reaction, which usually presents a latent period of 24 to 48 hours. It is of great interest to note below that within the first 24 hours, there are distinct histological abnormalities in the epithelium of the small intestine, yet this is always a period free from any clinical evidence of injury but showing a slight rise in urinary nitrogen.

The appearance of clinical intoxication coincides with the actual initial cell disintegration which is so evident in the epithelium of the small intestine in the second 24 hours. It is of interest that the crypt epithelium suffers first and shows evidence of actual necrosis and solution before any change is apparent in the epithelium covering the villi. This may well indicate some undetermined differences in

function as well as in histology between these two types of epithelial cell. The epithelium of the villi does not show much disturbance until the 3rd day, when the clinical intoxication is also well marked with bloody diarrhea and bile-stained vomitus.

The severity of the intoxication on the 3rd and 4th days coincides with the disappearance of much of the epithelium all through the small intestine. One may choose to explain this reaction as due to absorption of toxic substances and *invasion of bacteria from the lumen of the injured intestine.* These factors may be in part responsible for the intoxication, but we believe there are other possibilities which must be considered. Some of these are to be reviewed more in detail in subsequent papers of this series.

EXPERIMENTAL OBSERVATIONS.

All materials used in this study are obtained from dogs killed under anesthesia and autopsied at once. Tissues are fixed at once in formaldehyde (10 per cent) and stained usually by the routine hematoxylin-eosin method. Control tissues from normal dogs are fixed and prepared in exactly the same manner. A minimum of mechanical handling before fixation is necessary in this work. Routine examination of all organs in gross and histologically is carefully performed, but in the protocols normal findings are often omitted from the record.

Experiment 1. Lethal Dose of Roentgen Rays over Abdomen. Killed after 2 Hours.

Dog 20–33.—Normal, mongrel, adult, male; weight 32.5 pounds. Dog fasting for 4 days previous to radiation. Roentgen ray exposure over abdomen in four quadrants; no screens used, but usual 2 mm. aluminum filter. In all, 480 milliampere minutes given; E. M. F. of 89 kilovolts; current strength 8 milliamperes; skin-target distance of 10 inches. Exposure given between 3 and 5 p. m. 6 p. m. Dog sacrificed by bleeding under ether anesthesia.

Autopsy.—Performed at once. All viscera examined carefully and found to be normal, except for the kidneys, which show a few scars in the cortex. The intestinal tract shows no congestion and no abnormalities of any sort.

Histological Sections.—All organs and tissues examined in routine sections. There are very few abnormalities. The kidneys show arteriosclerosis and dense scars; the prostate shows some hyperplasia. Spleen and mesenteric glands show a good deal of nuclear fragmentation in centers of follicles.

Gastrointestinal tract at first sight is negative, but careful comparison with sections of normal control dogs convinces one that some of the nuclei in the crypt epithelium of the duodenum and jejunum do show slight abnormalities. These nuclei are fuzzy looking and the chromatin is beginning to show very slight evidence of injury. These changes are much more noticeable in the lymph glands of this region.

Experiment 2. Lethal Dose of Roentgen Rays over Abdomen. Killed after 24 Hours.

Dog 19–130.—Apr. 15, 2 p.m. Normal, old adult, male mongrel; weight 45 pounds. Dog fasting for 3 days previous to radiation. X-ray exposure over abdomen, 450 milliampere minutes; 90 kilovolts; current strength 7.5 milliamperes; spark-gap 9 inches; 2 mm. aluminum filter; skin-target distance 10 inches. Dog given morphia (½ gr.) before exposure.

Apr. 16, 2 p. m. Dog normal in all respects; chloroform anesthesia and exsanguination.

Autopsy.—Done at once. All viscera examined with care and are normal with the following exceptions: *Small intestine* is tightly contracted and contains only bile-stained fluid. Duodenum and jejunum show the mucosa thrown up into longitudinal folds whose crests are specked with ecchymoses. There are a few small scattered ecchymoses all through the small intestine. The mucosa is not markedly congested but is unusually *bile-stained* and this may be due to actual vital staining of the injured cells.

Histological Sections.—Spleen and mesenteric lymph nodes show cell necrosis in lymph follicles and numbers of phagocytes. All of small intestine shows definite nuclear changes in the epithelium of the crypts. Nuclei are pale or show fragmented chromatin material. There is a definite invasion of polymorphonuclear leucocytes. The epithelium of the tips of the villi shows no distinct change as yet.

Experiment 3. Lethal Dose of Roentgen Rays over Abdomen. Killed after 48 Hours.

Dog 19–101.—Apr. 9. Normal, adult, female mongrel; weight 33 pounds. Dog fasting for 4 days and through experiment. X-ray exposure over abdomen, 450 milliampere minutes; 90 kilovolts; current strength 7.5 milliamperes; spark-gap 9 inches; 2 mm. aluminum filter; skin-target distance 10 inches. Dog given morphia (½ gr.) before exposure. Thorax and pelvis screened by lead.

Apr. 10. Dog is normal.

Apr. 11. Dog bright and active, but vomits some bile-stained mucus at 2 p. m. Ether anesthesia and sacrifice at 4 p. m.

Autopsy.—At once. All organs examined with care and are normal except where noted otherwise. Stomach contains a little bile-stained mucus, but mucosa is normal. Small intestine contains a little bile-stained mucus and a little bright

red blood in one section; the mucosa in general is pale, but shows scattered ecchymoses. There are longitudinal folds whose crests are pink and dotted with ecchymoses. There are few congested patches in the ileum. Colon is spastic but mucosa normal.

Histological Sections.—Stomach mucosa is normal. There is a sharp line of transition from normal stomach to abnormal duodenal mucosa. Here the duodenal crypts show fragmented nuclei and cell degeneration. A few wandering cells are in evidence. Mitotic figures indicate an effort on the part of the epithelium to regenerate. There is some edema of the submucous tissues. The epithelium covering the villi appears uninjured. This condition is general throughout the small intestine. There is a sharp line of demarcation at the ileocecal valve and the colon mucosa seems practically normal. Colon shows occasional crypts presenting degenerating epithelial cells and invasion by wandering cells. Ovary shows degenerating ova with chromatin fragmentation.

Experiment 4. Lethal Dose of Roentgen Rays over Abdomen. Killed after 3 Days.

Dog 19–50.—Feb. 11. Normal, adult, female spaniel; weight 32 pounds. Dog fasting for 2 days before radiation and through experiment. X-ray exposure over abdomen, 340 milliampere minutes; 97 kilovolts; current strength 7.5 milliamperes; spark-gap 9½ inches; 2 mm. aluminum filter; skin-target distance 10 inches. Thorax and pelvis shielded by lead. Morphia (¼ gr.) before exposure.

Feb. 12. Dog lively; a little frothy vomitus in afternoon.

Feb. 13. Dog less active; blood-stained stools and bile-stained vomitus.

Feb. 14. Dog weak, much bright blood in stools, which contain mucus and clots, very foul smelling; vomiting less frequent. Ether anesthesia and sacrifice.

Autopsy.—At once. Viscera all examined carefully and found normal unless otherwise noted. Kidney: slight scarring of cortex. Pancreas: a few tiny fat necroses close to bile duct. Stomach contains blood-tinged, bile-stained mucus, but its mucosa seems normal. Small intestine contains a little bloody mucus and it is irregularly contracted. *Duodenum* shows a dark, glassy, red mucosa which is thin and in places wrinkled. The mucosa has lost its velvety appearance. This is explained by the histology. The rest of the small intestine shows much the same picture but the surface is mottled and some areas are much more abnormal than others. Peyer's patches are rather conspicuous, due to the thinning of the mucosa. This change in the mucosa begins sharply at the pylorus and ends as sharply at the ileocecal valve. Colon: mucosa is in general pale but there are a few ecchymoses.

Histological Sections.—Unless noted are negative. Lymph follicles in mesenteric glands and spleen show evidence of injury and removal of damaged cells. Ovary shows degenerating and necrotic ova. Bone marrow—lumbar vertebra—shows few marrow cells remaining and much cell debris in large phagocytes. Stomach: some cells in the mucosa appear slightly abnormal but there is nothing definite. *Duodenum:* much of the epithelium covering the villi and lining the

crypts has disappeared. In many places, the villi appear as naked polyps, a little swollen and containing dilated capillaries. The crypts are often filled with cell debris and wandering cells. At times mitotic figures are seen in the remaining epithelium. Wandering cells are conspicuous in all this area—usually polymorphonuclears. There is a noticeable edema of the submucosa. The whole structure of the mucosa has collapsed because of the destruction of the epithelium. Some sections show a mucosa which is almost normal. This "escape" of these areas will be discussed later. *Jejunum* and *ileum* show the same extensive destruction of the epithelium with consequent collapse of the familiar structure of the mucosa. Many sections would scarcely be recognized as coming from the small intestine. In places the mucous surface is covered with an exudate of red and white cells with fibrin. Some areas show only a moderate injury, corresponding to the gross appearance.

Experiment 5. Lethal Dose of Roentgen Rays over Abdomen. Killed after 4 Days.

Dog 19–80.—Jan. 21. Adult, female mongrel; weight 20.5 pounds. Dog fasting for 2 days previous to radiation and subsequently. X-ray exposure over abdomen, 350 milliampere minutes; 95 kilovolts; current strength 7.5 milliamperes; spark-gap 9½ inches; skin-target distance 10 inches; 2 mm. aluminum filter. Dog given morphia (¼ gr.) before radiation. Thorax and pelvis screened with lead.

Jan. 22. Dog active and normal.

Jan. 23. Dog less active, but no other clinical symptoms.

Jan. 24. Dog sick with soft stools and usual vomitus.

Jan. 25. Dog prostrated; stools contain fresh blood and clots; temperature is falling and dog becoming comatose; ether anesthesia and sacrifice 90 hours after radiation.

Autopsy.—At once. Unless otherwise stated viscera are normal. Mesenteric glands are enlarged, moist, and congested. Stomach contains blood-tinged mucus and its mucosa is normal. Small intestine contains a little bloody fluid and a few worms. The mucosa in many loops is dark red and glassy, specked with ecchymoses. Other areas are more nearly normal and show the velvety texture of the mucosa. The jejunum seems to be less uniformly injured than the duodenum and ileum. Longitudinal folds with hemorrhagic crests are found in all parts of the small intestine. Colon shows a pale mucosa specked with occasional ecchymoses.

Histological Sections.—The changes noted in this autopsy are almost identical with those described in detail in Experiment 4. The epithelial cells of the mucosa are conspicuous by their absence, and there is more evidence of mitosis and early repair on the part of the few epithelial cells which remain in the injured crypts. There is a moderate grade of edema of the submucosa and some invasion by wandering cells. The congested capillaries in the naked villi are the obvious

source of the blood in the intestinal contents. Occasional thrombi are noted in these vessels. Stomach and colon show minimal changes. In similar experiments one may find considerable epithelial degeneration in the crypts of the colon mucosa but it is obvious that this epithelium is much more resistant than is that of the ileum.

DISCUSSION.

We may review the positive features of this Roentgen ray reaction in dogs day by day following the exposure. The first 24 hours show no clinical disturbance. These dogs would pass as normal on the most critical examination. The urinary nitrogen shows a slight but constant increase above normal. The body cells, however, show some definite changes. The spleen and lymph glands show readily recognized cell injury and nuclear fragmentation within 2 hours. The epithelium of the crypts of the small intestine also within 2 hours shows distinct but faint nuclear changes. Within 24 hours, this intestinal epithelium shows nuclear fragmentation and cell injury together with a wandering cell reaction (leucocytes and macrophages). The ova in the ovary show the familiar nuclear reaction of degeneration.

After 2 hours, the small intestine may show a distinct flushing of the mucosa. This may or may not be due wholly to the x-rays, but it may be noted as perhaps similar to the erythema of the skin which may be noted a few hours after an erythema skin dose. The skin may not show distinct injury (burn) for many days after this initial reaction to injury by the x-rays. Apparently the various body cells have individual peculiarities in their reaction to toxic doses of the x-rays, especially as regards the latent period. Compare leucocytes, intestinal epithelium, and skin epithelium.

Within the second 24 hours after a toxic dose of the x-rays, we see many distinct changes from normal. Usually there is definite clinical abnormality, although at times this may be very slight. As a rule, there is loss of appetite and activity. The dog often vomits a little mucus and passes a soft stool or two. Autopsy at this time shows an intestinal tract which may appear normal except for a few ecchymoses in the small intestine. There may be abnormal bile staining of the mucosa, due in part perhaps to the presence of dead epithelium. The intestine in general is apt to be spastic and reacts to the stimuli with very vigorous contractions. This irritation of the muscle coats

is probably the result of the early inflammation beginning in the mucosa and submucosa. This spastic tendency, we believe, explains the longitudinal folds which are so conspicuous in the 3rd and 4th days with their crests marked out by congestion and hemorrhage.

Histologically, in the second 24 hours, we find a number of important changes, practically all in the small intestine. The epithelium of the crypts shows severe injury with nuclear destruction and even cell necrosis and desquamation, so that the crypt lumina are filled with cell debris and wandering cells. The epithelium of the villi still remains to all intents normal. The wandering cells are not yet conspicuous in the submucosa and are usually polymorphonuclears. There are a little submucous edema and occasional extravasation of red cells. These changes are conspicuous from the pylorus to the ileocecal valve. The colon shows occasional small patches in which the crypts show similar but less advanced injury.

The third 24 hour period is one of violent clinical disturbance and frank anatomical changes, mostly limited to the small intestine. The dog is constantly retching and frequently vomiting a thick mucoid bile-stained material. There is usually much fluid diarrhea, often with blood and mucus. Tenesmus may be much in evidence. Smears from fecal material show numbers of red and white blood cells, epithelial cells, and bacteria. This period gives marked anatomical changes in a typical case. The entire small intestine from pylorus to ileocecal valve looks raw, red, and thinned out. Some areas may escape and appear relatively normal. We believe these areas of severe injury of the mucosa are to be explained by overlapping areas of exposure. They may also be in the uppermost coils of intestine in the abdomen.

The histological material is of great interest but we cannot review the details. There is almost complete loss of crypt and villous epithelium, and the mucosa collapses into an atypical matrix of villi and empty crypts. Some sections are almost unrecognizable. Villi appear as naked polyps with congested capillaries. A few crypts may contain occasional epithelial cells which are large and contain mitotic figures—an attempt at early repair. The bloody fecal contents are easily explained by this picture. In spite of these raw areas we do not observe the intense inflammatory reaction which might be

expected. There are a little edema and a distinct increase in wandering cells. We believe this point is of much interest as regards the common conception of the intestinal epithelium as the sole barrier between the intestinal bacteria and the blood and lymph stream. This point will be taken up again in detail with a study of its bacteriology.

The fourth 24 hour period is much like the third but shows increasing intoxication. Death usually follows on this day. There is great loss of weight and strength. Vomitus and diarrhea continue unabated and the dog finally goes into coma preceding death. The histology is much like that described for the 3rd day, but evidences of epithelial repair may be more conspicuous and of fascinating interest to the histologist.

It is a common observation that lymph follicles and Peyer's patches show less injury than do the neighboring epithelial cells of the mucosa. Our sections would indicate that the epithelium of the small intestine is at least as sensitive to the x-rays as are the lymphocytes of the same area. In fact, by a large dose of the x-rays, we can actually destroy all of the epithelium, while many clusters of lymphocytes still remain. It is possible, of course, that lymphocytes in different parts of the body have varying susceptibilities to radiation, so that this observation in the intestine may not be applicable to the lymphocytes elsewhere in the body.

It may be noted at this time that a recent publication of Denis, Martin, and Aldrich (1) differs considerably in its interpretation of the abnormalities noted after long x-ray exposures. Rabbits were used and in our experience are not as favorable animals for this work, although the changes are similar. In their discussion, Denis, Martin, and Aldrich suggest that these epithelial changes in the intestine are "the result rather than the cause of the intoxication" and not primarily responsible for the intoxication. We are confident that our experiments give convincing proof that this epithelial injury in the small intestine is due to the x-rays and is in itself responsible for the progressive and lethal intoxication.

SUMMARY.

Roentgen radiation in lethal dosage, given over the abdomen of a normal dog, is followed by a physiological reaction of remarkable uniformity.

The first 24 hour period following the exposure is negative clinically and anatomically, but histologically we see frank changes in the bone marrow, spleen, lymph glands, and ovaries. There are definite nuclear changes with degeneration in the crypt epithelium of the small intestine.

The second 24 hour period shows slight clinical disturbances of gastrointestinal nature (vomitus and diarrhea). The mucosa of the small intestine shows scattered ecchymoses, but the histology of the small intestine is important. The necrosis of the crypt epithelium may be almost complete, while the epithelium of the villi remains practically intact. There are a little edema and invasion of wandering cells.

The third 24 hour period shows increasing clinical disturbance with vomiting and bloody diarrhea. Anatomically the small intestine from the edge of the pylorus to the rim of the ileocecal valve looks raw, red, and inflamed. The crypt and villous epithelium has in large part vanished, leaving a collapsed framework of the mucosa showing a little edema and invasion of wandering cells.

The 4th day marks the peak of the intoxication, and death usually takes place at this time, preceded by coma. Anatomically and histologically the picture is like that of the 3rd day. There is more evidence of mitosis and efforts of repair on the part of the intestinal epithelium.

The stomach is not concerned in this reaction, but the colon may show evidences of a slight injury. The colon is obviously much more resistant than is the small intestine.

We believe the evidence is conclusive that the injury done the epithelium of the small intestine is wholly responsible for the stormy clinical picture and fatal intoxication.

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ROENTGEN RAY INTOXICATION.

III. Speed of Autolysis of Various Body Tissues after Lethal X-Ray Exposures.

The Remarkable Disturbance in the Epithelium of the Small Intestine.

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The question of Roentgen ray injury and certain modifications of intracellular ferments has been reviewed by Hall and Whipple (1). We must admit that the various methods of studying intracellular ferments are at present very crude and probably give mere hints as to what goes on within the cell. These ferments are accustomed to their normal environment which is very carefully balanced by the presence of the circulating fluids which remove waste products and furnish necessary supplies to the cells. The reaction of these ferments in a mass of dead or dying cells cut off from circulatory influences may or may not have any significance as to the true physiological function of these ferments.

We have submitted much evidence of true cell injury involving both nucleus and protoplasm following x-ray exposures. We submit below evidence to show that cell autolysis in the epithelium of the small intestine is profoundly modified by x-ray exposures. Some investigators go so far as to claim that primary injury of cell ferments is a fact and is in itself responsible for subsequent reactions and cell death. But how can one tell that this modification of cell autolysis is due to a primary ferment influence or some other physiological disturbance which influences the ferments secondarily? When the investigator gives this explanation of primary cell ferment reaction, he is indeed in distress when he attempts to explain the great differ-

ences in the effect of the x-rays on different cell structures. Our methods permit of a distinction in cell and nuclear structure between these cells which react so very differently to the x-rays. But present methods do not permit us to recognize a great multitude of individual cell ferments in the parenchymatous organ cells. Compare the cells of the bone marrow, intestinal mucosa, pancreas, and liver. They are all rich in ferments but they react very differently indeed to the x-rays. One must be very conservative in the analysis of data concerning intracellular ferments and deductions drawn from such experiments.

It was pointed out by Hall and Whipple (1) that the intestinal epithelium in radiated dogs was subject to rapid postmortem autolysis, so much so, that a few hours after the death of the radiated animal very little of the epithelium could be demonstrated. These workers also indicated that the crypt epithelium was more abnormal in this respect than the tip epithelium. With these observations in mind, a series of autolysis experiments was undertaken using sections of organs from the series of dogs described in Paper II. In this series an attempt is made to demonstrate the various changes in cell autolysis observed at various periods following a lethal x-ray exposure.

Method.

A series of dogs was exposed to 350 to 480 milliampere minutes of radiation diffusely over the abdomen. These experiments are described in the preceding paper. Dogs were sacrificed under chloroform or ether anesthesia at various periods of 2, 24, 48, 72, or 96 hours after exposure to radiation. A set of sections of each organ for control is fixed immediately in 10 per cent formaldehyde. Five sets of sections, each section about 3 to 5 gm. in weight, are cut from each organ immediately after death of the animal. Each section is placed, while still warm, in a bottle containing approximately 150 cc. of warm 0.9 per cent sodium chloride solution previously saturated while warm by shaking for an hour with an excess of chloroform. All the bottles are then warmed to 37°C., corked, placed in an incubator at 37°C., and the tissues allowed to autolyze. A set of sections is removed at 4, 8, 10, 12, and 24 hours respectively and immediately fixed in 10 per cent formaldehyde. There is no putrefactive odor

present in the autolysis bottles, even after 24 hours autolysis of intestinal sections, indicating that the growth of putrefactive bacteria has been inhibited by the chloroform and suggesting that the autolysis is due to the endogenous ferments. There are no free chloroform globules present in the autolysis bottles, it being completely held in solution. The odor of chloroform is still quite strong after 24 hours autolysis. Several sets of experiments were done with sodium chloride solution without chloroform, but these were found to be very irregular, probably due to variation in the outgrowth of bacteria.

Organs from three normal unradiated dogs are treated to the same routine and used as controls.

Histological Method.

The autolysis sections are studied with regard to the effect of radiation upon the rapidity of autolysis *in vitro*. Gradations of autolysis are based upon the intensity of staining of the nuclei, the integrity of the cell outline and the cell contents, and the complete digestion of the cell itself. In designating changes for the purposes of constructing curves indicating the speed of autolysis, the following criteria are used:

0 means no autolysis perceptible.

A or + means cell nuclei are pale and washed out, as compared with the control of that organ—slight autolysis.

B or ++ means nuclear structure is lost, run together, or indistinct—well advanced autolysis.

C or +++ means the nuclear outline is shadowy and indistinct. There may appear to be nothing left but nuclear debris in the space occupied by the cell—marked autolysis.

D or T means total autolysis; the nuclei are gone, though the basement membranes are usually intact. At this stage, there is nothing left but pale fibroblasts, polymorphonuclears, endothelial cells of the blood vessels, and the fibrillar intracellular matrix.

It would seem that these arbitrary periods of autolysis are irregular in sequence but these periods were established by preliminary experiments and seemed to us to give the required information. In plotting these findings, it also serves our purpose to consider the individual stages (or extent) of autolysis and the individual period of

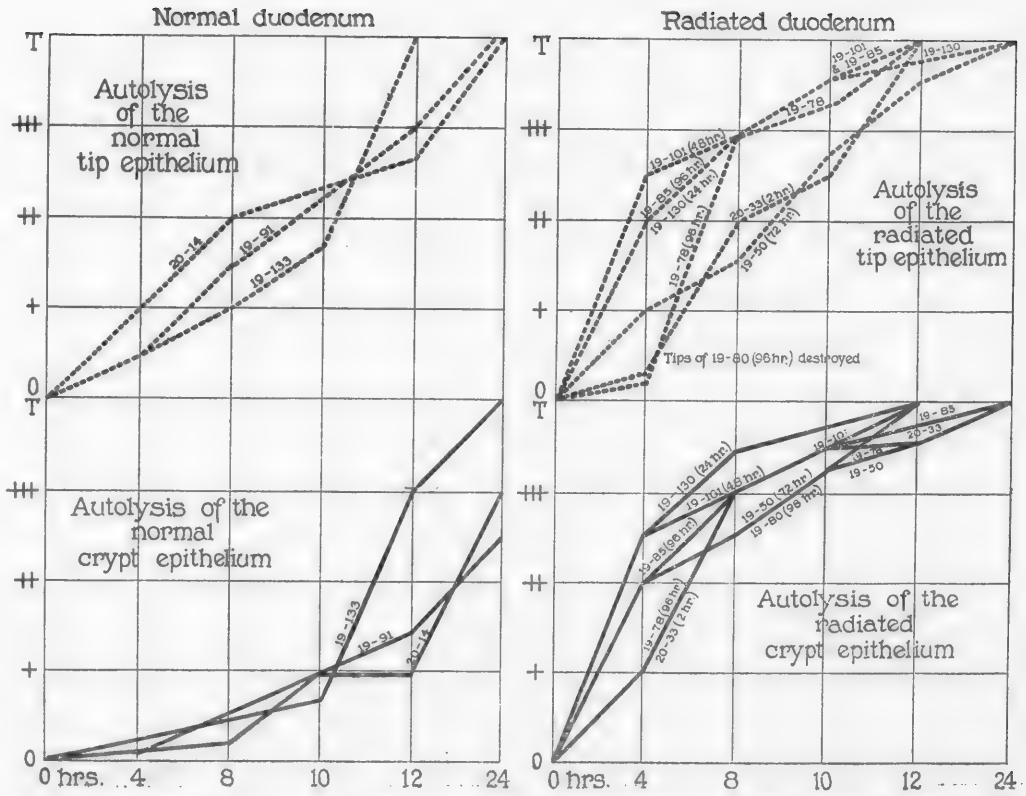
autolysis as equal and numerically progressing units upon the coordinates. It is evident that if all of the constants are equal and presented in the same manner, the experimental results are being compared upon a satisfactory basis. Therefore, although all of our units are arbitrary, they are constant throughout and any consistent variation in the plotted curves may be considered as demonstrating changes produced by the experimental procedure (exposure to radiation).

EXPERIMENTAL OBSERVATIONS.

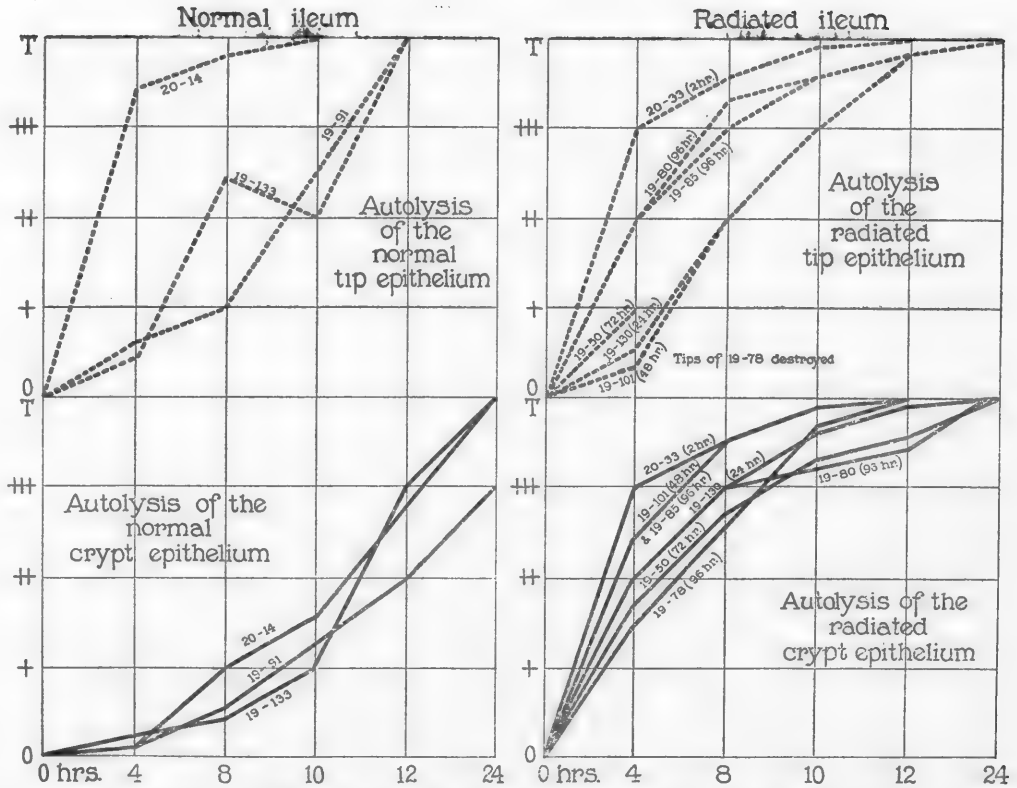
All the histological material for this study was fixed in 10 per cent formaldehyde and stained by the usual hematoxylin-eosin method, using paraffin sections. About 600 slides were studied and reviewed at least three separate times as unknown specimens to prevent any bias from knowledge of sequence of events in autolysis periods. The grades of autolysis have been established and described in the preceding paragraph. Curves of the various tissue reactions were established in form similar to Text-figs. 1 and 2. These many charts cannot be published but the statements made below are based on a study of such charts and sections. Much of the more important data can be studied in Table I.

We may note that certain cells common to organs and tissues are not much, if at all, disturbed by these long x-ray exposures. Such are fibroblasts and mature connective tissue cells, endothelial cells and macrophages, muscle tissues, and certain white blood cells. All these tissues are resistant to autolysis in the control and radiated animals and may appear as very little changed even by the long 24 hour period of autolysis at 37°C. Some, but not all, of these cells are supposed to be poor in ferments.

The more specialized cell types of the abdominal viscera are our chief concern. In the gastrointestinal tract, we differentiate between the tip epithelium which covers the villus and the crypt epithelium which is more of the secreting cell type. In the spleen and mesenteric lymph nodes, we have made a rather broad distinction between the pulp and the Malpighian bodies; in the pancreas between the isles of Langerhans and the remainder of the parenchyma. In the kidney, the proximal convoluted tubules are contrasted with the straight



TEXT-FIG. 1. Comparison of the autolysis of the duodenum of normal and of radiated dogs.



TEXT-FIG. 2. Comparison of the autolysis of the ileum of normal and of radiated dogs.

TABLE I.

Autolysis during Periods of Hours as Indicated.

	Control.					2 hrs. after x-rays.					24 hrs. after x-rays.					48 hrs. after x-rays.					72 hrs. after x-rays.				
Hrs. autolysis.	4	8	10	12	24	4	8	10	12	24	4	8	10	12	24	4	8	10	12	24	4	8	10	12	24
Duodenum crypt	0	0	A	A	C	A	C	C	D	—	B	C	D	D	—	B	C	D	D	—	B	C	D	D	—
" villi	A	A	B	C	D	"	B	"	C	D	"	"	C	"	—	"	"	C	"	—	A	B	C	"	—
Jejunum crypt	0	0	A	B	C	B	C	D	D	—	"	"	D	"	—	"	"	D	"	—	B	C	"	"	D
" villi	0	A	B	C	D	0	A	B	C	D	A	"	C	"	—	A	B	C	"	D	—	—	—	C	—
Ileum crypt	0	"	A	B	C	C	C	D	D	—	B	"	D	"	—	B	C	D	"	—	B	C	D	D	—
" villi	A	B	B	C	D	B	"	"	"	—	A	B	C	"	—	A	B	C	"	—	A	—	—	—	—
Colon	0	A	"	"	"	A	B	C	"	—	B	C	"	"	—	"	C	"	"	—	"	B	C	D	D
Stomach	A	"	"	"	"	B	C	D	"	—	A	B	"	"	—	"	B	"	"	D	"	"	"	"	"
Liver	"	B	"	"	"	"	"	C	"	—	"	C	"	"	—	"	"	"	"	—	"	"	"	"	"
Pancreas	0	A	"	"	"	"	"	"	"	—	B	"	"	"	—	"	"	"	C	D	"	"	A	B	"
Spleen	A	B	"	"	"	"	"	"	"	—	C	"	D	"	—	B	C	"	D	"	"	"	B	"	"
Kidney	0	A	"	"	C	A	B	B	C	C	0	A	A	B	C	A	A	B	C	C	B	"	C	C	"

Degree of autolysis indicated as follows (see complete description under Histological method): 0 means zero autolysis. A means slight autolysis. B means well advanced autolysis. C means marked autolysis. D means total autolysis.

tubules and the glomeruli, mainly because of their difference in autolysis; in the liver, the parenchyma cells are contrasted with the bile ducts.

In the normal intestine, the autolytic digestion of the mucosa starts in the tips of the villi and progresses fairly slowly and evenly to the bases of the crypts. Even at the end of 24 hours, the crypt epithelium may be fairly well preserved while the tip epithelium is almost totally autolyzed. In the *radiated animal*, on the other hand, *the crypt epithelium autolyzes at a greater speed than the tip epithelium* and is practically completely autolyzed in 10 to 12 hours or earlier, while even after 12 hours of autolysis the tip epithelium in the same animal may be fairly well preserved (compare Text-figs. 1 and 2).

Discussion of Experiments.

Normal Autolysis.—Three normal dogs are sacrificed under anesthesia. Sections of abdominal viscera are autolyzed at body temperature in 0.9 per cent sodium chloride solution saturated while warm with chloroform as described above. The usual course of digestion is from without inward and the progress is steady and continuous. The digestion in the gastrointestinal sections progresses from the villi to the bases of the crypts. Comparisons are made of the penetration of the autolysis as well as of the progress of cell digestion.

Stomach: The chief and parietal cells of the gastric mucosa are more susceptible to autolysis than are the tip cells. The former are autolyzed rapidly and are totally digested at the end of 10 hours. The tip cells autolyze somewhat more slowly and are completely digested in 24 hours.

Duodenum, jejunum, ileum, and *colon* are quite similar in the type of their autolysis. The tip epithelium is the first to autolyze. In these control experiments the time may vary from 10 to 24 hours before completion of the digestion. Considerable variation is to be noted in this reaction of the epithelium of the villi. The autolysis of the crypts is usually delayed 4 to 8 hours, after which it proceeds regularly though usually not to completion in 24 hours (Text-figs. 1 and 2).

Spleen pulp is found to autolyze 4 to 8 hours faster than the Malpighian bodies, which, as a rule, are not completely autolyzed by 24 hours. Their centers are fairly well preserved at 12 hours. The pulp at this time is usually pretty well digested.

Pancreas digests pretty well as a unit. The islands of Langerhans autolyze on the average 2 to 4 hours more slowly at first than the parenchyma but later the speed varies considerably so that they may totally digest in 10 hours, or only partly digest in the full 24 hours.

Kidney convoluted tubules are very sensitive to autolysis. Usually they are completely digested in 8 or 10 hours. The remainder of the *kidney parenchyma* consisting of the straight and collecting tubules and the glomeruli is more slowly digested, the full 24 hours usually being required for their complete autolysis.

Liver parenchyma and bile capillaries autolyze as a unit.

Autolysis of Radiated Organs.—Seven normal dogs are exposed to large amounts of radiation, given diffusely over the abdomen, and sacrificed at definite periods after exposure. Autolysis specimens are studied (as above) in regard to the progress of digestion in the specialized cells of the various organs.

Stomach epithelium, though the speed of autolysis may seem to have increased slightly, is digested well within normal limits. This is true for the whole series of dogs which were killed 2, 24, 48, 72, and 96 hours after radiation.

Intestinal epithelium shows a striking change from the controls. The speed of autolysis of the tip epithelium in each case is practically within normal limits. The great change has occurred in the speed of the autolysis of the crypt epithelium. The curves in Text-figs. 1 and 2 illustrate this very well.

The speed of autolysis of the crypt epithelium is rapid and must be due to the immediate effect of radiation upon these cells. The most pronounced increase in the speed of autolysis occurs within the first 2 hours after exposure to radiation. The change produced in these cells, or what is left of them, is permanent up to the time of the death of the animal 4 days later. Even after a lapse of 4 days, these cells autolyze faster than the normal crypt epithelium. These curves correspond remarkably well, indicating that the increase in autolysis is due to a change in the cell protoplasm which persists

at least for 4 days after radiation. In all of the animals exposed to radiation, the crypt epithelium of the small intestine is completely autolyzed in 12 hours in the duodenum, ileum, and jejunum, while in the experiments with normal animals, the crypt epithelium is rarely autolyzed completely in 24 hours and usually the autolysis is only well advanced by that time.

Colon shows an increase in the speed of autolysis of the crypt epithelium and possibly of the tip epithelium, though the latter is less pronounced. The effect of the radiation seems to decrease or wear off slightly, for the speed of autolysis is somewhat slower and approaches the normal in the 3rd and 4th days after exposure to radiation. This may be connected with the fact that there is also relatively little injury to the colon, anatomically or histologically. The most pronounced increase in the speed of autolysis in the colon epithelium occurs, therefore, within the first 48 hours.

Spleen shows an apparent increase in the speed of autolysis, especially in the first 24 hours after exposure to the x-rays. The 72 hour curve is within normal limits, while both the 48 and the 96 hour curves show an increased speed in autolysis.

Pancreas shows a slight but definite increase in the speed of autolysis, especially during the first 48 hours after exposure. The 96 hour curves seem to indicate also that the speed of autolysis is increased, but the 72 hour curves are irregular as in the spleen series. The islands of Langerhans autolyze as a rule somewhat faster than the pancreatic parenchyma.

Kidney does not show changes in the speed of autolysis after exposure to radiation. The convoluted tubules are totally autolyzed in 8 hours. The other tubules and the glomeruli, while autolyzing more slowly, apparently digest at the normal rate. This would suggest that radiation has little effect upon the kidney.

Liver parenchyma shows a definite increase in the speed of autolysis, with a maximum in the specimens removed 2 hours after the radiation. The 24 and 48 hour experiments show less increase above normal.

DISCUSSION.

Many observers have studied ferment changes *in vitro* in normal organs or tumor tissues exposed to x-rays before the unit period of autolysis. Much of this work has been reviewed by Hall and Whipple and need not be discussed. Our experiments indicate that the spleen, liver, and pancreas taken from a radiated animal will show a more rapid autolysis than similar tissues from control normal animals under similar conditions of autolysis. The kidney shows no such change by the methods used. We may recall that the spleen shows obvious histological injury after exposure to the x-rays, but such changes are lacking in the liver, pancreas, and kidney.

The stomach and colon are much more resistant than the small intestine, but we shall publish experiments to show that large and permanent gastric and colon ulcers may be produced by the x-rays.

The *duration of the injury* done the body cells is of much interest to us. We note that abnormal autolysis of the spleen, liver, and pancreas is most striking within 24 hours after the radiation. After 48 hours, there is a distinct decrease in this reaction as observed in dogs killed 72 and 96 hours following the lethal dose of x-rays. In the small intestine, it is easy to demonstrate the abnormal autolysis in dogs even 96 hours after the x-ray exposure. This indicates in a sense that these cells are suffering from a persistence of the injury exerted by the x-rays, even during the 4th day after the exposure. It is significant that the clinical symptoms always reach a crisis on the 4th day. If this period is survived, the dog usually goes on to recovery.

We may note in passing that the cell necrosis and injury in the intestine as in the skin is associated with a minimal wandering cell reaction. This point deserves further study. We shall record also the fact that ulcers caused by the x-rays are as stubborn in healing in the stomach and intestine as is so well recognized in the skin.

SUMMARY.

Exposure to large doses of x-rays will cause notable increase in the speed of autolysis of the crypt or secretory epithelium of the dog's small intestine. These changes can be demonstrated readily in

material obtained from dogs killed 2, 24, 48, 72, or 96 hours after the initial radiation (Text-figs. 1 and 2).

In the radiated dogs the secretory crypt epithelium of the small intestine autolyzes first and the epithelium of the villi last, while the reverse is true in the normal control small intestine. These abnormalities of autolysis associated with lethal Roentgen ray exposures can be demonstrated for the small intestine over the whole 4 day period subsequent to radiation.

The colon shows little change and the stomach no demonstrable changes in autolysis under like conditions. The kidney likewise is negative.

The spleen, lymph glands, liver, and pancreas show a moderate increase in speed of autolysis in tissues taken from radiated animals within 48 hours of the initial exposure.

What the significance of this disturbance of cell ferments in the intestinal mucosa may be, we cannot pretend to say. At least these observations strengthen one's confidence in the profound functional disturbance of this important intestinal epithelium—a disturbance which we believe is responsible for the clinical abnormalities and fatal intoxication.

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A STUDY OF RENAL FUNCTION IN ROENTGEN RAY INTOXICATION.

Resistance of Renal Epithelium to Direct Radiation.

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In an earlier publication (Hall and Whipple (4)), the noticeable clinical similarity between intestinal obstruction and systemic x-ray intoxication has been pointed out. We have recorded elsewhere (10, 11) an interesting transient disturbance of renal function in the intoxication due to intestinal obstruction and in certain proteose intoxications. This disturbance of renal function is considerable and plays an important part in certain of the clinical reactions (intestinal obstructions). We believe this is the first instance of a well established functional depression of renal secretory function totally unassociated with any abnormalities of renal structure. Because of this peculiar disturbance of renal function in certain proteose intoxications, we took up a study of renal function in systemic x-ray intoxication with the keenest interest. We may say at once that our experiments show a totally different picture, with the renal function uninvolved in the toxic disturbance of general x-ray intoxication. There is a minimal disturbance of renal function by the direct action of very large doses of the hard x-rays but no anatomical evidence of cell injury. We feel confident that the clinician may use the x-rays over the kidney areas with confidence that renal tissue is resistant to the hard Roentgen rays. Our experiments indicate that the kidney epithelium is much more resistant to x-ray injury than is the epithelium of the small intestine.

Inspection of the literature shows that work has been done to determine possible injury of the kidney by the x-rays. However, most of the reports deal with the morphological changes produced or consist of clinical observations made by use

of the older, less reliable means of determining the presence of functional disturbances. Warthin (18) noted pathological changes in the kidneys of several leucemia patients who had received x-ray treatment before death and reports finding definite changes in the tubular epithelium following prolonged percutaneous irradiation of the kidneys of white mice and rats. Rosenstern (13), Linser and Helber (9), Schleip and Hildebrand (15), von Jaksch (6), Heymann (5), Přibram and Rotky (12), and others likewise claim to have found evidence of an x-ray nephritis. It has been thought by many that the inability of injured kidneys to do their work efficiently must be considered as an essential factor in the constitutional x-ray reaction. While Edsall and Pemberton (3) considered the primary intoxication to be quite independent of any direct effect of the x-rays upon the kidneys, they suggest that the kidneys may become overtaxed by the increased work thrown upon them to such an extent that their failure may secondarily intensify the symptoms of intoxication.

There are many workers, however, who have failed to find any definite clinical or postmortem evidence of an x-ray nephritis. Among those who have reported negative findings may be mentioned Krause and Ziegler (8) and Buschke and Schmidt (2). The latter workers incised the abdominal wall and exposed the kidneys of a number of rats directly to large doses of the x-rays. The animals died a few weeks later, two of them only showing albumin in the urine and pathological changes in the structure of the kidneys at autopsy. Since in these two cases the changes might well have been due to some complication following the operations, these workers concluded that the renal epithelium is very resistant to the x-rays and that consequently there is no such thing as an x-ray nephritis. Warthin, whose work is more recent and freer from complications, admits that the anatomical changes were so slight in his experimental animals that they might be overlooked unless special histological technique is employed. Krause (7) in 300 cases finds no albumin or leucocytes in the urine after radiation. Stephan (16) in a small series of cases claims to cure anuria and produce a diuresis in 24 hours by means of $\frac{1}{8}$ to $\frac{1}{4}$ of an erythema dose over the kidneys. This brief reference to the work previously done on the subject suffices to show the decided lack of agreement among those who have given any attention to it. There is obvious need for a thorough functional study of the problem with employment of the comparatively accurate methods now available for measuring the functional activity of the kidneys under conditions of carefully controlled x-ray administration.

Method.

Normal adult dogs were used exclusively in all of the experiments. The animals were selected after being under observation for a period of several days during which time the functional tests to be used in the subsequent experiments were performed on them. The data

recorded in this preliminary examination on each dog serve as a standard with which to compare results obtained in subsequent experiments. At the end of this fore period the animal was exposed to a carefully measured dose of x-rays, following which the renal function was determined daily for a period of a week or more.

The hard Roentgen rays of the Coolidge tube were used exclusively, the softer rays being filtered out with 2 mm. aluminum filters. In a very few cases only did we have leakage with slight tendency to pigmentation and denudation of the skin. All exposures were percutaneous and the distance from the target (anticathode) to the surface of the animal's body measured in all cases 25 cm. The ammeter was made to read at approximately 8 milliamperes in most of the experiments, although currents as high as 10 milliamperes and as low as 5 milliamperes were employed in a few instances. Our work confirms the observation of Hall and Whipple (4) that in dogs the toxicity of the hard rays increases with the increase in spark-gap. Our experience has shown that a 9 inch spark-gap is a convenient one for producing the desired experimental intoxication and that this required an E. M. F. of about 90 kilovolts. With a few exceptions, this voltage was employed.

In a part of the experiments moderate sized sublethal doses of x-rays were given at intervals over a considerable period of time, a short rest on a mixed diet being given between exposures. In the remainder, huge and frequently fatal doses were given singly or in succession. It was considered that between these extremes no impairment of function, whether prompt or delayed in its appearance, could escape detection. In order to understand the nature or the mechanism of any disturbance which might be observed, different regions of the body were exposed separately in the various experiments.

For a period of 1 to 3 days preceding and 5 to 6 days subsequent to the radiation, the dogs were given only water. During resting intervals, the dogs received the usual kennel mixed diet of table scraps, cooked meat, bread, rice, bones, and so forth.

The methods chosen for the measurement of the renal function were those employed by us in studies on the renal function in intestinal obstruction (10); namely, the phenolsulfonephthalein elimination test (14) and that of determining the capacity of the kidneys to ex-

crete urea. The former method was carried out according to the procedure outlined by its authors, the Duboscq colorimeter being used for the determination of the dye. For the latter we made a daily determination of the blood urea in all experiments and determined in many cases the ratio

$$\frac{\text{Urea in 1 hr.'s urine}}{\text{Urea per 100 cc. of blood}}$$

after the intravenous injection of comparatively large amounts of urea in saline solution. A decrease in this ratio indicates a definite impairment of kidney function as shown by Addis and Watanabe (1). The tests were made after the exposure to the x-rays exactly as in the fore period except that in cases of extreme intoxication with resulting loss of fluid from the body, more fluid was given intravenously in order that the loss might be made up as far as possible. The urea of both the blood and the urine was determined by the Van Slyke-Cullen modification of the Marshall urease method (17). Morphine sulfate ($\frac{1}{8}$ to $\frac{1}{4}$ gr. doses) was administered subcutaneously 1 hour before the animal was placed upon the x-ray table to insure quiet during the period of exposure.

EXPERIMENTAL DATA.

The experimental data assembled below consist of three types of experiments. The first includes a group of observations on several different animals in which *sublethal doses of x-rays* were given at intervals over relatively long periods of time. In these experiments, the thorax and the abdomen, including the kidneys, were alternately exposed. The purpose of this series was to ascertain whether or not repeated x-ray treatment over a long period of time is capable of producing any gradual changes in the renal function. Many have feared the possibility of cumulative action resulting in permanent damage to the kidneys.

Dog 19–85 (*Tables I and II*). *Autopsy Record.*—Dog sacrificed under ether anesthesia 4 days after the last radiation, Apr. 5. The kidneys in gross are quite normal. Bladder normal. Histological sections show normal stroma, vessels, and glomeruli. There are no scars. Convoluted tubules are normal.

TABLE I.

Large Sublethal Doses of X-Rays Given over Abdomen and Thorax. Renal Function Unimpaired.

Dog 19–85. Adult female spitz.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Nov. 30	24.9	23	75	Normal.
Nov. 30	190 milliampere minutes, x-rays over abdomen and kidneys; 90 kilovolts; 10 milliamperes.			
Dec. 1	24.7	22	71	Normal.
" 3	24.2	36	74	"
" 5	23.7	34	54	Slight diarrhea.
" 6	23.2	36	55	No diarrhea. Normal.
" 7	22.7	40	63	Normal.
Dec. 16	220 milliampere minutes, x-rays over thorax and neck; 90 kilovolts; 10 milliamperes.			
Dec. 17	24.3	32	62	Normal.
" 18	23.9	30	69	—
" 19	23.1	42	57	—
" 20	23.0	31	52	—
" 21	22.9	30	67	Normal.
Dec. 31	350 milliampere minutes, x-rays over abdomen and back; 90 kilovolts; 8 milliamperes.			
Jan. 1	22.6	—	67	Normal.
" 2	22.0	22	67	—
" 3	21.8	20	71	—
" 4	21.2	23	63	Normal.
" 5	20.8	30	67	—
" 6	20.4	28	63	—
Feb. 4	300 milliampere minutes, x-rays over abdomen and back; 95 kilovolts; 8 milliamperes.			
Feb. 8	23.0	34	63	Normal.
" 9	23.0	30	63	—
" 10	22.6	28	66	—
" 11	22.4	24	64	—
" 12	22.2	25	63	—

Mixed diet in intervals between experiments; no food for 5 days following exposures.

It will be seen from Tables I to IV that the experiments of this group are a unit in showing that the excretory function of the kidneys, as measured by their ability to eliminate a foreign substance such as phenolsulfonephthalein or the normal urinary constituent, urea, is not disturbed by this procedure. It is evident that with the doses given there is no sign of any cumulative effect being produced, whether the thorax or abdomen or both be exposed.

TABLE II.

Large Sublethal Doses of X-Rays Given over Abdomen and Thorax. Renal Function Unimpaired.

Dog 19–85. Adult female spitz.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Dec. 21	9.00–10.00	—	—	30	—	—	Normal.
	10.00–12.00	20	3.59	262	13.7	67	—
Mar. 4	350 milliampere minutes, x-rays over thorax; 95 kilovolts; 5 milliamperes.						
Mar. 7	9.00–11.00	—	—	28	—	63	Normal.
" 8	—	—	—	27	—	64	—
" 9	10.00–12.00	20	3.78	270	14.0	67	Normal.
Apr. 1	350 milliampere minutes, x-rays over abdomen; 105 kilovolts; 5 milliamperes.						
Apr. 4	12.00–2.00	—	—	39	—	65	Moderate intoxication.
" 5	3.00–5.00	20	3.42	278	12.3	—	Same condition.

The second type of experiment is represented by the observations made on Dogs 19–127 and 19–140 (Tables V to VIII) in which a lethal dose of the x-rays was given over the abdomen with the kidneys and the remainder of the body protected from the direct rays by the leaden sheets. This type of experiment was designed to demonstrate whether or not a poisonous substance capable of injuring the kidneys is produced in the irradiated gastrointestinal tract and carried by the blood stream to the kidneys. Reference to the tables shows that the function of the kidneys remains entirely normal after the exposure,

TABLE III.

Repeated Sublethal Doses of X-Rays over Thorax and Abdomen. Renal Function Unchanged.

Dog 19–78. Adult female Airedale. (Autopsy, see Table XV.)

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Nov. 23	28.5	30	68	Normal.
Nov. 25	190 milliampere minutes, x-rays over thorax and neck; 85 kilovolts; 5 milliamperes.			
Nov. 26	28.1	18	63	Normal.
" 27	27.5	22	57	—
" 28	27.4	20	60	—
" 29	27.1	—	58	—
" 30	26.5	33	58	—
Dec. 1	26.4	28	60	—
" 2	26.4	23	68	No food since exposure.
" 5	26.0	20	60	Mixed diet.
" 10	27.5	—	62	—
Dec. 10	190 milliampere minutes, x-rays over abdomen and back; 90 kilovolts; 5 milliamperes.			
Dec. 11	27.5	40	59	Normal.
" 12	27.0	40	59	—
" 13	26.2	40	54	—
" 14	26.0	41	59	—
" 15	25.7	36	54	—
" 16	24.9	22	59	—
Dec. 27	240 milliampere minutes, x-rays over thorax; 90 kilovolts; 5 milliamperes.			
Dec. 28	26.6	36	64	Normal.
" 29	26.5	47	64	—
" 30	26.3	50	62	—
" 31	25.5	43	63	—
Jan. 1	25.0	33	59	—

Dog not fed for day or two before exposure and for 5 days after each radiation.

although the dose of x-rays produced profound intoxication and death. The evidence against any indirect action of the x-rays upon the kidney function or upon the structure of the kidney is very clear-cut. The blood urea showed a definite increase in both cases, but this was due

to accelerated tissue destruction rather than to retention, as is shown by the fact that the urea ratio (Tables VI and VIII) remained normal

TABLE IV.

Repeated Increasing Doses of X-Rays over Thorax and Abdomen. Renal Function Undisturbed.

Dog 19–50. Young adult female. (Autopsy, see Table XVI.)

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Dec. 5	30.40	28	74	—
Dec. 5	210 milliampere minutes, x-rays over thorax; 90 kilovolts; 10 milliamperes.			
Dec. 6	29.50	22	72	Normal.
" 7	29.30	31	66	—
" 8	28.20	31	63	—
" 9	27.00	34	55	—
" 10	26.40	34	63	—
" 12	26.50	—	—	—
" 14	26.50	28	70	—
" 15	27.10	33	—	—
Dec. 16	210 milliampere minutes, x-rays over abdomen and back; 90 kilovolts; 10 milliamperes.			
Dec. 17	27.15	37	56	Normal.
" 18	27.00	31	68	—
" 19	26.70	34	65	—
" 20	25.40	30	63	—
Dec. 31	350 milliampere minutes, x-rays over thorax; 90 kilovolts; 7.5 milliamperes.			
Jan. 1	26.70	—	72	Normal.
" 2	26.50	28	67	—
" 3	26.00	26	61	—
" 4	25.50	22	61	—
" 5	25.40	24	63	—
" 6	25.30	20	66	—
" 7	25.00	21	63	—

even when additional urea was injected intravenously. The results indicate the absence of any hypothetical nephrotoxic substance in the blood during the period of intoxication, and thus point out a

TABLE V.

Lethal Dose of X-Rays over Abdomen with Kidneys Shielded. Renal Function Unchanged.

Dog 19–127. Adult male mongrel.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
June 9	28.4	32	74	
" 10	28.3	30	72	Normal.
June 10	504 milliampere minutes, x-rays over abdomen, kidneys completely shielded; 90 kilovolts; 8 milliamperes.			
June 11	27.8	27	76	Normal.
" 12	26.3	35	67	Quiet.
" 13	26.1	44	68	Dull and weak.
" 14	25.5	46	66	Intoxication increasing.
" 15	—	53	59	Grave intoxication and collapse. Death in night.

TABLE VI.

Lethal Dose of X-Rays over Abdomen with Kidneys Shielded. Renal Function Unchanged.

Dog 19–127. Adult male mongrel.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
June 9	8.50– 9.50	—	0.160	32	5.0	—	—
	10.00–11.00	20	2.382	240	9.9	74	Normal.
	11.00–12.00	—	1.856	201	9.2	—	—
June 10	504 milliampere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.						
June 14	8.45– 9.45	—	0.144	46	3.1	—	—
	10.00–11.00	20	2.215	237	9.3	66	Severe intoxication.
	11.00–12.00	—	1.693	192	8.8	—	—

TABLE VII.

Lethal Dose of X-Rays over Abdomen with Kidneys Shielded. Renal Function Unchanged.

Dog 19–140. Young adult female collie.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
July 7	21.7	34	70	—
" 8	20.5	32	72	Normal.
July 8	450 milliampere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.			
July 9	19.7	30	70	Normal.
" 10	19.3	34	63	Dull and weak.
" 11	19.0	43	63	Severe intoxication; bloody diarrhea; death in night.

TABLE VIII.

Lethal Dose of X-Rays over Abdomen with Kidneys Shielded. Renal Function Unchanged.

Dog 19–140. Young adult female collie.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
July 8	7.55– 8.55	—	0.160	32	5.0	—	—
	9.00–10.00	20	3.028	244	12.4	72	Normal.
	10.00–11.00	—	2.353	219	10.7	—	—
July 8	450 milliampere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.						
July 11	8.30– 9.30	—	0.206	43	4.8	—	—
	10.00–11.00	20	2.744	240	11.4	68	Typical x-ray intoxication.
	11.00–12.00	—	2.366	213	11.1	—	—

difference between this type of intoxication and that of acute intestinal obstruction in which a transient impairment of kidney function is constantly found.

TABLE IX.

Large Doses of X-Rays Directly over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 19–117. Adult female setter.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
July 28	38.8	32	67	Normal.
July 28	456 milliampere minutes, x-rays directly over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.			
July 29	38.8	30	68	Normal.
" 30	38.4	29	66	—
" 31	38.0	27	66	Bread and milk diet.
Aug. 1	37.6	28	63	Dog a little sick.
" 2	37.4	24	63	—
" 3	37.8	—	—	—
" 4	37.4	26	48	—
" 5	37.0	33	32	—
" 6	36.4	—	39	—
" 10	—	33	49	Normal.
" 11	36.5	33	56	—
Aug. 12	512 milliampere minutes, x-rays over both kidneys with other parts shielded; 90 kilovolts; 8 milliamperes.			
Aug. 13	36.3	26	59	—
" 14	36.2	30	—	Dog fed; good condition.
" 15	36.0	30	55	—
" 16	36.1	—	56	—
" 17	36.2	32	48	—
" 18	36.0	38	45	Small amount of vomitus.
" 19	36.0	—	42	Moderate diarrhea.
" 20	35.2	32	58	Clinical improvement.
" 21	35.0	—	—	Normal.

Dog 19–127 (Tables V and VI). Autopsy Record.—Dog died in night of 4th day after exposure to a lethal dose of x-rays given over abdomen with shielding of the kidneys. Autopsy next morning. Kidneys and bladder normal in gross. Histological sections show some postmortem changes in the epithelium of the

convoluted tubules. The stroma and glomeruli are normal. There are no scars. The vessels are filled with blood.

The third type of experiment is represented by the series of observations on Dogs 19–117, 19–129, and 18–115 (Tables IX to XIV).

TABLE X.

Large Dose of X-Rays Directly over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 19–117. Adult female setter.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
July 14	12.50–1.50	—	0.170	30	5.6	—	—
	2.00–3.00	20	1.799	158	11.4	74	Normal.
	3.00–4.00	—	1.323	142	9.3	—	—
July 15	456 milliampere minutes, x-rays over thorax and neck; 90 kilovolts; 8 milliamperes.						
July 18	8.45– 9.45	—	0.160	27	5.9	—	—
	10.00–11.00	20	1.826	159	11.5	67	Normal.
	11.00–12.00	—	1.357	144	9.4	—	—
July 28	456 milliampere minutes, x-rays over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.						
Aug. 11	2.50–3.50	—	0.160	33	4.8	—	—
	4.00–5.00	20	1.505	173	8.7	56	Not clinically sick.
	5.00–6.00	—	1.182	163	7.3	—	—
Aug. 12	512 milliampere minutes, x-rays over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.						
Aug. 18	1.45–2.45	—	0.174	38	4.5	—	—
	3.00–4.00	20	1.342	179	7.4	45	Dull. Small amount vomitus.
	4.00–5.00	—	1.316	167	7.8	—	—

In these the kidneys were exposed to huge doses of the x-rays directly while the remainder of the abdomen and the thorax were completely shielded. Large doses were repeatedly given in the case of the first animal while large single doses only were given in the other cases.

TABLE XI.

Large Dose of X-Rays over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 19–129. Adult male mongrel.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Aug. 6	67.9	29	66	Normal.
" 7	67.4	28	67	—
Aug. 8	512 milliampere minutes, x-rays over thorax; 90 kilovolts; 8 milliamperes.			
Aug. 10	66.7	26	66	Normal.
" 12	65.0	29	63	—
" 14	64.8	25	64	Mixed diet.
Aug. 19	512 milliampere minutes, x-rays directly over kidneys, with other parts shielded; 90 kilovolts; 8 milliamperes.			
Aug. 19	63.0	29	67	Normal.
" 22	61.7	38	53	Slightly dull.
" 24	59.7	46	44	Little vomitus.
" 26	59.3	44	54	Slightly dull.
" 28	59.0	32	58	Normal.

TABLE XII.

Large Dose of X-Rays over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 19–129. Adult male mongrel.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Aug. 19	8.40– 9.40	—	0.142	29	4.9	—	—
	10.00–11.00	20	1.368	143	9.5	66	Control.
	11.00–12.00	—	1.083	129	8.4	—	—
Aug. 19	512 milliampere minutes, x-rays over kidneys with abdomen shielded; 90 kilovolts; 8 milliamperes.						
Aug. 26	—	—	—	47	—	—	—
	1.00–2.00	20	1.124	161	6.9	47	Slightly dull
	2.00–3.00	—	0.933	150	6.2	—	—

Dog 19–129 showed no signs of intoxication, whereas No. 19–117 showed a slight reaction and No. 18–115 was markedly intoxicated. It is probable that in the case of No. 18–115 a small portion of the intestine was included in the area of exposure. It is to be noted that the doses given over the kidneys in this group of experiments were at least 25 per cent greater than the lethal dose over the abdomen.

TABLE XIII.

Large Dose of X-Rays over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 18–115. Adult female bulldog.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Aug. 23	21.5	30	68	Normal.
" 24	20.2	27	66	"
Aug. 27	512 milliampere minutes, x-rays over kidneys with remainder of body shielded; 90 kilovolts; 8 milliamperes.			
Aug. 28	19.4	32	62	Normal.
" 29	19.2	31	54	—
" 30	19.0	33	58	Normal.
" 31	—	—	58	Slightly dull.
Sept. 1	18.3	52	40	Quiet; vomitus and tarry stools.
" 2	17.9	43	—	Tarry stools and vomitus.
Sept. 3	300 milliampere minutes, x-rays over abdomen alone; 90 kilovolts; 8 milliamperes.			
Sept. 4	17.2	36	53	Vomits food; tarry stools.
" 5	16.7	44	46	Weak and dull.
" 8	—	—	—	Found dead in cage.

Dog 19–117 (*Tables IX and X*). *Autopsy Record.*—Dog sacrificed under ether Aug. 24 and autopsy done at once. Kidneys and bladder normal in gross. Histological sections show normal glomeruli, vessels, and stroma. There are no scars. Convoluted tubules show normal epithelium. There are a few pale hyaline casts in the collecting tubules of the pyramids.

Dog 19–129 (*Tables XI and XII*). *Autopsy Record.*—Dog sacrificed with ether anesthesia Aug. 29 and autopsy done at once. Kidneys show definite chronic nephritis with a large cyst in the left kidney. There is a chronic granular cystitis. Histological sections show a marked cellular pyelitis in both kidneys. There are

a few cellular scars in the cortex and some large fibrous scars (arteriosclerotic) involving a considerable area of the cortex and showing tubular and glomerular destruction. There is no evidence of acute nephritis. Outside the scar areas, the glomeruli and tubules appear to be quite normal. This picture is often found in the kidneys of old dogs and cannot in any way be related to the x-ray exposures. This abnormal kidney seems to be no more susceptible to radiation than the normal kidney.

Dog 18–115 (Tables XIII and XIV). Autopsy Record.—Dog found dead in cage Sept. 8 and autopsy done at once. Kidneys are normal in gross. Histological sections show normal glomeruli and vessels. There are a few cellular scars in cortex. The convoluted tubules show a slight postmortem change, but otherwise the tubules are normal.

TABLE XIV.

Large Dose of X-Rays over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 18–115. Adult female bulldog.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Aug. 24	2.30–3.30	—	0.153	27	5.6	—	—
	4.00–5.00	20	2.562	197	13.1	66	Control.
	5.00–6.00	—	1.998	181	11.0	—	—
Aug. 27	512 milliampere minutes, x-rays over kidneys with remainder of body shielded; 90 kilovolts; 8 milliamperes.						
Sept. 1	1.30–2.30	—	0.203	52	3.9	—	—
	3.00–4.00	20	2.014	217	9.2	40	Dog is weak. Tarry stools and vomitus.
	4.00–5.00	—	1.683	179	9.4	—	—

Tables IX to XIV inclusive, in which the data of these experiments are recorded, show that the direct exposure of the kidneys to massive doses of the x-rays gives rise to a moderate but definite depression of the kidney function. In Tables XV and XVI are shown the results of two experiments differing slightly from the foregoing in that the kidneys and the intestines were together exposed to large doses of x-rays, the thorax only being protected. A definite decrease in the 2 hour output of phenolsulfonephthalein and a corresponding drop in the urea ratio indicate a moderate impairment of renal function.

TABLE XV.

Lethal Dose of X-Rays over Kidneys and Abdomen.
Renal Function Moderately Impaired.

Dog 19–78. Adult female Airedale.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Dec. 7	1st	—	—	24	—	—	—
	2nd	20	1.826	152	12.0	69	Control.
	3rd	—	1.594	131	12.1	—	—
Mar. 18	350 milliampere minutes, x-rays over abdomen and kidneys; 95 kilovolts; 8 milliamperes.						
Mar. 20	—	—	—	32	—	55	Slightly dull.
" 21	—	—	—	45	—	40	Intoxication progressing.
Mar. 22	1st	—	—	69	—	—	—
	2nd	20	1.861	258	7.2	38	Marked intoxication; bloody diarrhea.
	3rd	—	1.443	239	6.0	—	—

Dog killed under ether at end of observation.

TABLE XVI.

Lethal Dose of X-Rays over Kidneys and Abdomen.
Renal Function Slightly Impaired.

Dog 19–50. Young adult female.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Feb. 10	1st	—	0.240	29	4.8	—	—
	2nd	20	1.983	180	11.0	66	Control.
	3rd	—	1.529	156	9.8	—	—
Feb. 11	340 milliampere minutes, over abdomen and kidneys; 95 kilovolts; 8 milliamperes.						
Feb. 13	—	—	—	42	—	56	Dull; vomitus and diarrhea.
Feb. 14	1st	—	0.328	78	4.2	—	—
	2nd	20	2.146	280	7.66	40	Frequent diarrhea and vomiting.
	3rd	—	1.543	249	6.19	—	—

From the results of the experiments outlined above we assume that the direct action of the x-rays upon the kidneys is responsible for this depression of activity.

Dog 19–78 (*Tables III and XV*). *Autopsy Record.*—Dog killed under ether at end of experiment given in Table XV. Autopsy done at once but only the renal findings given here. Kidneys are normal in gross. Histological sections show normal glomeruli, vessels, and stroma. There are no scars. Convoluted tubules are normal. The epithelium of the loops of Henle shows a frothy looking protoplasm but this picture is seen in control dogs.

Dog 19–50 (*Tables IV and XVI*). *Autopsy Record.*—Dog killed under ether anesthesia at end of last experiment (Table XVI) and autopsy at once. Only the renal findings are given here. Kidneys and bladder normal in gross. Histological sections show normal glomeruli and vessels. There are a few small cellular scars in the cortex. Tubules are normal.

Reference to the autopsy findings in the various dogs used in the experiments shows that no constant anatomical change is seen in the kidneys which would serve to distinguish them from a series of normal control kidneys chosen at random. We did not use the refined histological technique recommended by Warthin, but we are inclined to believe that much of the histological evidence submitted in favor of x-ray injury of the kidney is due to kidney abnormalities present *before the x-ray exposures*. Such abnormalities would be revealed by a careful study of suitable controls.

SUMMARY.

Our experiments give no support to the current belief that an x-ray nephritis may be produced by direct or indirect action of the hard Roentgen rays.

Moderate doses of x-rays given *repeatedly* over considerable periods of time have no demonstrable influence on renal function or renal structure.

With x-ray exposures of the abdomen and shielding of the kidneys fatal intoxication may be produced without the slightest disturbance of kidney function as measured by the ability of the kidney to eliminate phenolphthalein and urea.

Large doses of the x-rays given directly over the kidney may cause a slight but distinct lowering of renal function which lasts for a

period of a few days. We have been unable to recognize any corresponding histological change.

We feel that the usual therapeutic doses of the x-rays can be given over the kidneys without apprehension. The renal tissue is much more resistant to x-rays than is the epithelium of the small intestine.

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THE RELATION OF THE HYPOPHYSIS TO ANTIBODY PRODUCTION.

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The important part that many of the endocrine organs play in development and function suggests the possibility that they may also enter into the mechanism of the body which develops resistance to infection. Up to the present time there is little positive evidence that such is the case. It was felt, however, that certain of the more inaccessible glands of internal secretion, whose function in relation to immunity had not been studied, might yield valuable information, and this study of the pituitary gland was made as part of a general investigation into this field (6).

The experimental work so far has been chiefly of a physiological nature concerning the part the pituitary plays in growth, metamorphosis, sugar tolerance, and sexual activity, and its relation to acromegaly, dyspituitarism, and diabetes mellitus. The functions of the two parts of the gland have been elaborately defined (13, 16). More recently the interrelationship of this gland to the other ductless glands has been much discussed. That total hypophysectomy is incompatible with life[1] would seem to be proved by the work of Cushing and his collaborators (3, 4) and Paulesco (17). The striking physical changes in growth, development, nutrition (3), and general metabolism (2) with partial hypophysectomy (1) and by feeding pituitary gland extract (9, 10, 20) are now accepted. We have not been able to find, however, any direct, quantitative, experimental studies of the relation of the hypophysis to infectious diseases. Aschner (1) thought his partially hypophysectomized dogs succumbed more easily to disease than normal dogs, and certainly many contracted pneumonia. Cushing's experiments (3) seem to bear this out. There are, however, no clinical observations that would seem to indicate a lessened or increased resistance to infectious disease

[1] The work of Gemelli (8), Aschner (1), and Handelsmann and Horsley (10) disputes this, but their extirpation methods were so injurious and uncontrolled as to appear unreliable. Our own studies on the guinea pig agree with those of Paulesco and Cushing.

with pituitary disorders.[2] Gay and Rusk (7) in a study of the locus of antibody production draw no definite conclusion, but the evidence would seem to indicate that the spleen and the lymphatic system play the most important part. The work of Murphy and his associates (15), Hektoen (11), Hektoen and Curtis (12), and Morris and Bullock (14) appears to corroborate this view. The experiments of Topley (19) appear to disprove the contention that antibodies are formed at the point of inoculation.

Problem and Methods.

Guinea pigs were selected as the experimental animal because of their already standardized reactions in immunological studies. An operative technique was elaborated for partial hypophysectomy. In such animals the serum reactions of typhoid agglutination, hemagglutination, and hemolysis were studied. These studies were completed in the following series.

Series 1. The Production of Antibodies after Hypophysectomy.—Partial hypophysectomized, control operation animals and normal controls were immunized to *Bacillus typhosus*. Subsequent titers indicated the degree of immunity produced.

Series 2. The Effect of Hypophysectomy on Antibody Production.—This was measured by the change in height of the antibody curve. Guinea pigs were immunized to (1) *Bacillus typhosus* and (2) hen red blood corpuscles, and then submitted to partial hypophysectomy. Normal and control operation animals were used. Titration of agglutinins and hemolysins was carried out at intervals before and after operation.

Series 3. The Effect of Feeding and Injecting Pituitary Extract on the Production of Antibodies.—Animals were immunized, then fed continuously pituitary extract or submitted to repeated injection of the extract, and their subsequent titers determined.

Operative Technique.

Adult guinea pigs weighing from 300 to 400 gm. were used. In such animals the pituitary is relatively large, weighing about 0.025 gm. (Vanderburgh (21)). It lies in a shallow dural pocket and has the characteristic histologic appearance of other mammalian hypophyses. After some difficulties the following operative attack was chosen as giving an approach through which a definite portion of the

[2] Personal communication from Dr. Harvey Cushing.

gland could be removed and in which success was obtained in about 75 per cent of the animals used. The animal was placed on an electrically heated pad, kept at a fixed temperature, and fastened down to a small raised table. Anesthesia was induced by means of a fine rubber tube passed into the posterior pharynx through which a small pump forced ether vapor (5). Morphia 0.002 gm. and atropine 0.004 gm. were given 15 to 30 minutes before anesthesia. After shaving the head and cleaning the scalp with 1:1,000 bichloride a wet gauze covering was placed over the head and a sterile towel over the body of the animal. The gauze over the vertex of the skull was cut and an incision in the skin was made from just behind the eyes to the occipital protuberance and carried through to the bone. The tissue flaps on both sides were then dissected up and held away by the weight of a single hemostat. The parietal bone was perforated and all the bone over both hemispheres was removed as far anteriorly as the fronto-parietal synostoses and posteriorly to the occipital bone. On the right side the decompression was carried to the temporoparietal juncture and on the left well into the temporal bone. The dura was left intact and the longitudinal sinus which lies in it, free from the bone, remained uninjured. The dura was then incised low down in the left temporal region, a small spatula introduced beneath the temporal lobe, and the brain elevated and slightly dislocated towards the right side. (The removal of bone on the right side was done to permit this.) A view was thus obtained of the pituitary fossa, the small pinkish body lying surrounded on both sides and posteriorly by large venous sinuses. The stalk enters at the anterior end of the gland, running slantwise to the posterior region. The dura over the middle of the hypophysis was then nicked with a small hook knife and with a small double spoon forceps fragments of the gland were removed. Unless the sinus was injured, which was fatal, there was little hemorrhage. Warm normal saline solution on cotton pledgets was used for sponging and hemostasis. After removal of the gland fragment, the brain was allowed to return to its position and the skin carefully sutured with two layers of fine interrupted silk sutures. This was covered by a cocoon dressing. The operation consumed between 30 and 45 minutes.[3] Control animals in which the same operation, without removal of the pituitary, was performed, were prepared. The above procedure was carried out through the step in which the dura over the sella was cut and then closure completed, thus subjecting the control animals to approximately the same amount of trauma and anesthesia.

Total hypophysectomy proved fatal, the animals almost at once entering into a lethargic, partly comatose state, refusing food and water and dying in 24 to 48 hours. All fragments removed were

[3] The chief complication in recovered animals was an ocular palsy, the result apparently of injuring the third cranial nerve which lies practically on the hypophysis but which we attempted to push to one side before the final step of enucleation.

studied histologically, and no animals are reported in which there was not this proof that at least one-half of the gland, which always consisted of fragments of both pars anterior and posterior, was removed. Autopsy controls were used to establish further the efficacy of this method of attack. The surviving animals within 24 hours made excellent recoveries, but lost 50 to 100 gm. in weight in the first few days. After this they regained weight and appeared, except for a rare ocular palsy, as active and vigorous as the normal controls. In many cases the gain in weight was rapid and even above that of the normal animals.

Technique of the Serum Reactions.

Typhoid Agglutinins.—The Sen strain of *B. typhosus* was used.[4] It had been grown on artificial media for years but still combined a ready agglutinability with a high toxicity for guinea pigs. It was carried along throughout these experiments by repeated daily transplants on agar and bouillon with occasional plating to determine its purity. A vaccine was prepared by suspending the 24 hour growth in six Blake bottles in salt solution, killing the organisms by adding chloroform, drying them in a vacuum, and then grinding up the bacteria in a sterile mortar. Weighed quantities were then suspended in normal salt solution and tricresol was added for maintaining sterility. Experimentation with vaccine and living organisms determined that the greatest reaction was produced by the following inoculations given at 3 and 4 day intervals: first injection, 0.002 gm. of vaccine subcutaneously; second injection, 0.002 gm. intraperitoneally; third injection, $\frac{1}{10}$ of a 20 hour growth of living organisms on an agar slant intraperitoneally; and fourth injection, $\frac{1}{10}$ of a 20 hour growth of living organisms on an agar slant intraperitoneally. By the 10th day after the last injection agglutinin titers ranged from 1:1,000 to 1:6,000. In Text-figs. 1 and 2 the normal control curve represents the average of over twenty-five animals. In performing agglutination tests 1 drop of a salt suspension of a 24 hour growth of the bacteria on agar was added to 1 cc. of successive dilutions of serum. Serum was obtained by cardiac puncture, 4 cc. of blood being withdrawn, and the serum inactivated. Titration was recorded after 2 hours at 37°C., after 2 hours at room temperature, and after the tubes had been in the ice box over night. 4 cc. of blood proved sufficient for both agglutination and complement fixation tests. The latter tests, however, although completed with almost all animals used, are not reported since the concentration of complement fixation bodies was not sufficient to ensure reliable comparative results.

[4] This strain was obtained from Dr. Carroll G. Bull.

Hemagglutinins and Hemolysins.—Another series of guinea pigs with normal controls, partially hypophysectomized controls, and operation controls was immunized to hen red blood corpuscles for the study of hemagglutinins and hemolysins. At 4 day intervals all animals were given intraperitoneally 1 cc. of washed hen corpuscles made up to the original blood volume. At the end of 1 week serum was obtained and the quantity of reaction determined. In testing for hemagglutinins 1 drop of a 10 per cent suspension of washed hen corpuscles was added to 1 cc. volumes of successively diluted, inactivated serum. Readings were made as with the typhoid studies. For hemolysins the usual technique of the Wassermann reaction was followed with 0.25 cc. volumes of inactivated serum, fresh guinea pig complement 1:10, a 5 per cent suspension of hen corpuscles, and 0.5 cc. of saline solution. The tubes were then incubated for 1½ hours, being shaken after 30 minutes and 1 hour. Readings were made after 1½ hours and after standing in the ice box over night. The usual controls were performed with each test.

The several series were performed with the above methods and complement deviation studies were also made. The latter, however, are not reported because of the low and variable nature of the titers resulting; it appears probable that for this test insufficient antibodies are manufactured. Operative difficulties now and then caused the loss of valuable animals but a sufficient number was always used in each experimental unit to ensure a satisfactory number of complete studies. As a rule, the experimental unit consisted of two normal animals, two partially hypophysectomized animals, and two operative control animals for Series 1. Series 2 experiments, however, were usually started with eight guinea pigs, since the operation was completed only when an immunity had been established and titers determined, and unless sufficient animals were provided the loss of a guinea pig at operation would vitiate that particular experimental unit. Before commencing with typical experimental units, the operative technique was perfected and a supply of hypophysectomized and control operation animals provided. The agglutinin, hemolysin, and hemagglutinin reactions were studied in normal guinea pigs and the quantitative reaction was well standardized. The immunity production in partially hypophysectomized and operative control animals showed no appreciable difference in these preliminary studies. In the actual experimental units guinea pigs as near of a size and kind as possible were chosen. Because of the reported interrelation of the

pituitary body with the sex glands (9) and the reported increased reaction in menstruating women to typhoid vaccine, preliminary studies were made which ruled out any appreciable influence that such sexual periods might have on antibody production. Neither menstruation[5] nor the pregnant state appeared to have any influence on the antibody curve.

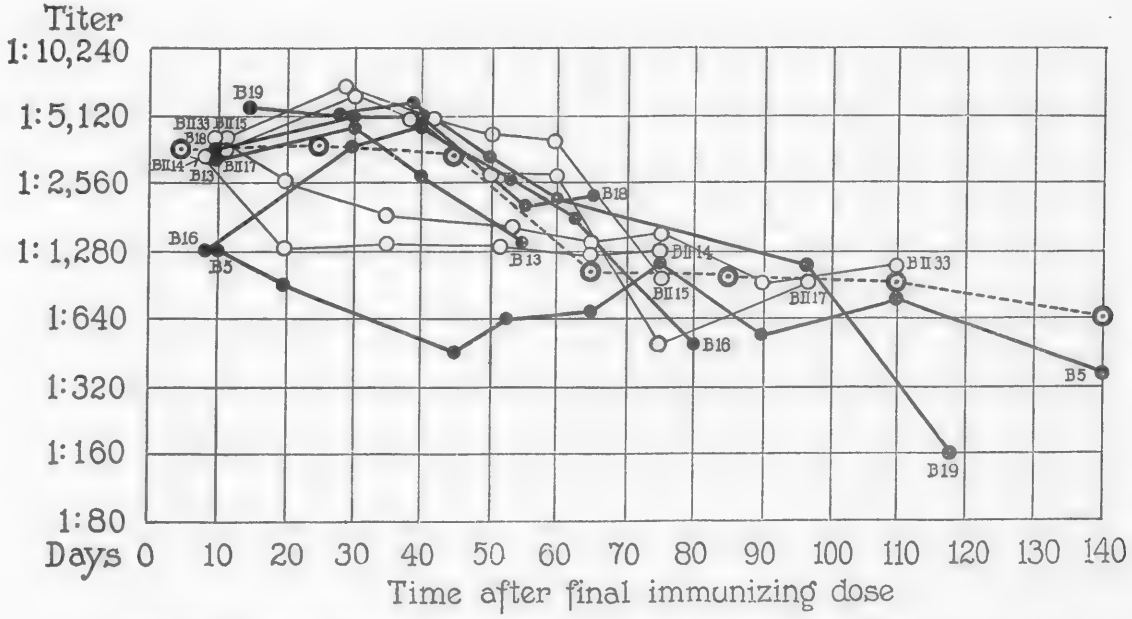
EXPERIMENTAL.

Series 1. The Production of Agglutinins after Hypophysectomy.—Guinea pigs, in which partial hypophysectomy[6] and a control operation[6] had been performed were immunized to *Bacillus typhosus* by the above method. Their agglutinin titers were recorded from the 7th to the 10th day after the last injection and followed every week or 2 weeks. Unoperated control animals were carried along in each experimental unit, but their course ran such a comparatively parallel curve to the large number of normal animals studied previously that an average line has been used for Text-figs. 1 and 2 in order to avoid confusion. Text-fig. 1 represents a group of hypophysectomized animals with operated controls compared to the normal average agglutinin curve. The general curve in the hypophysectomized and control operation animals shows a parallelism to the normal controls and needs no comment. The repetition of such experiments brought the same results. Partial hypophysectomy apparently has no influence on the subsequent production of typhoid agglutinins in guinea pigs.

Series 2. The Effect of Hypophysectomy on Antibody Production.—Three sets of experiments were performed. In Class 1 the agglutinin titer in animals immunized to *Bacillus typhosus* was studied before and after hypophysectomy, in Class 2 the hemagglutinin titer, and in Class 3 the hemolysin production. Normal and operated controls were used. For each experimental unit eight animals were used. All were immunized, two were saved for normal controls and three each submitted to partial hypophysectomy or the control operation.

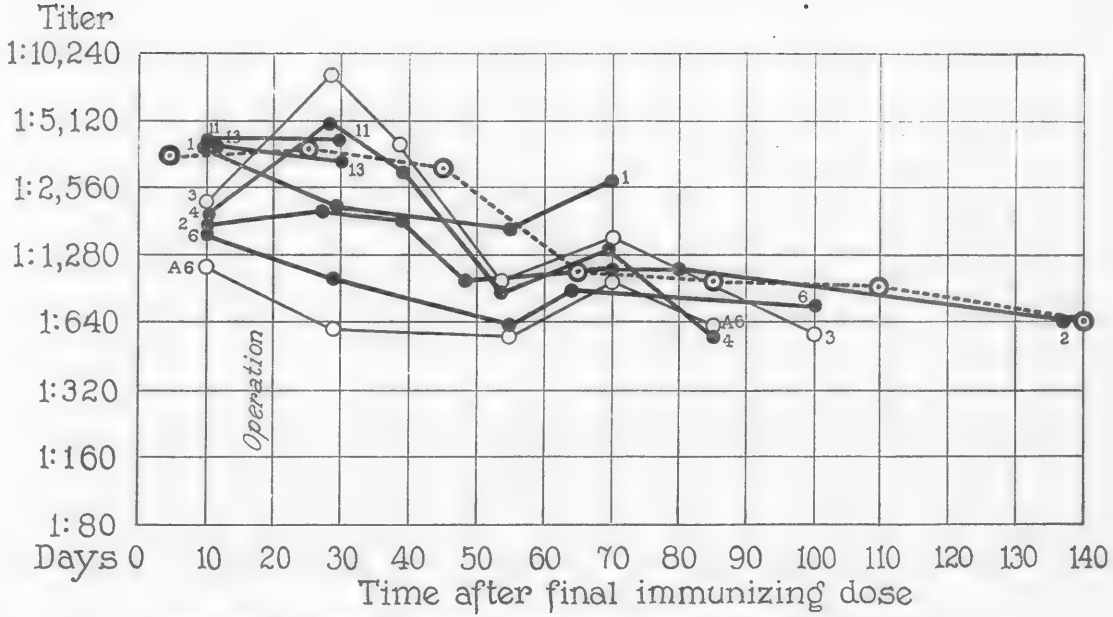
[5] The guinea pig has a definite menstrual cycle (18).

[6] At least 8 days after the operation, when the animals were entirely recovered.



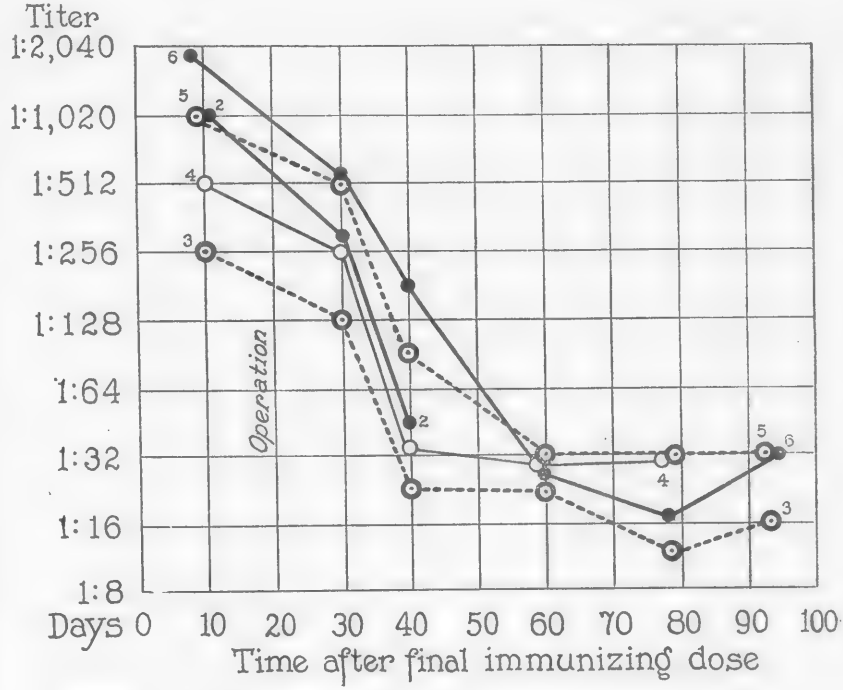
In Text-figs. 1 to 4 the following curves are used. ——— Partially hypophysectomized animals.
——— Control operation animals. ---------- Normal control animals.

TEXT-FIG. 1. Series 1. Typhoid agglutinin titers of animals immunized after hypophysectomy.



TEXT-FIG. 2. Series 2. Typhoid agglutinin titers before and after hypophysectomy.

Text-fig. 2 represents the study of such an experiment in Class 1, in which the animals were immunized to *Bacillus typhosus*. No special comment is necessary since all titrations fall practically within normal limits.



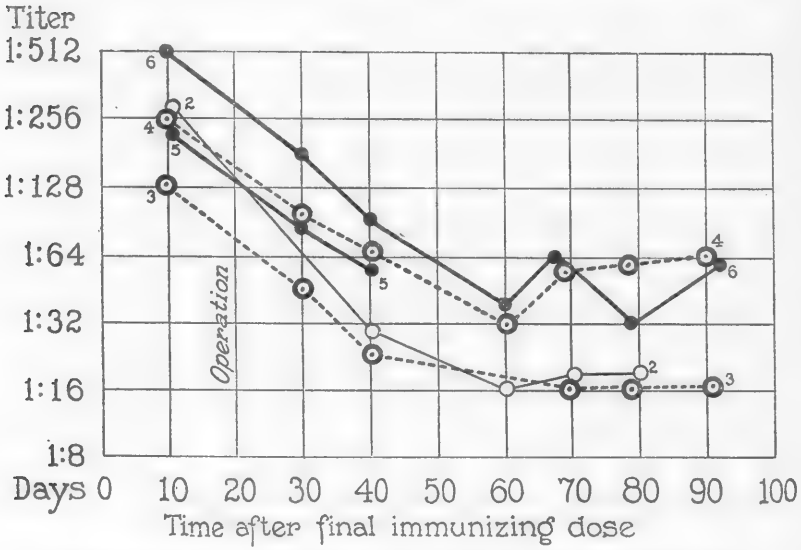
TEXT-FIG. 3. Series 2. Hemagglutinin titers before and after hypophysectomy.

Text-figs. 3 and 4 represent the studies in Classes 2 and 3. Here, too, the parallel of operated animals to normal animals is striking. Repeated experimental units showed no appreciable variation.

These experiments would seem to indicate that partial hypophysectomy has no influence on the maintenance of specific agglutinins for *Bacillus typhosus*, hemagglutinins, or hemolysins in the guinea pig.

Series 3. The Effect of Feeding and Injecting Pituitary Extract on Antibody Production.—In the feeding experiments six animals were

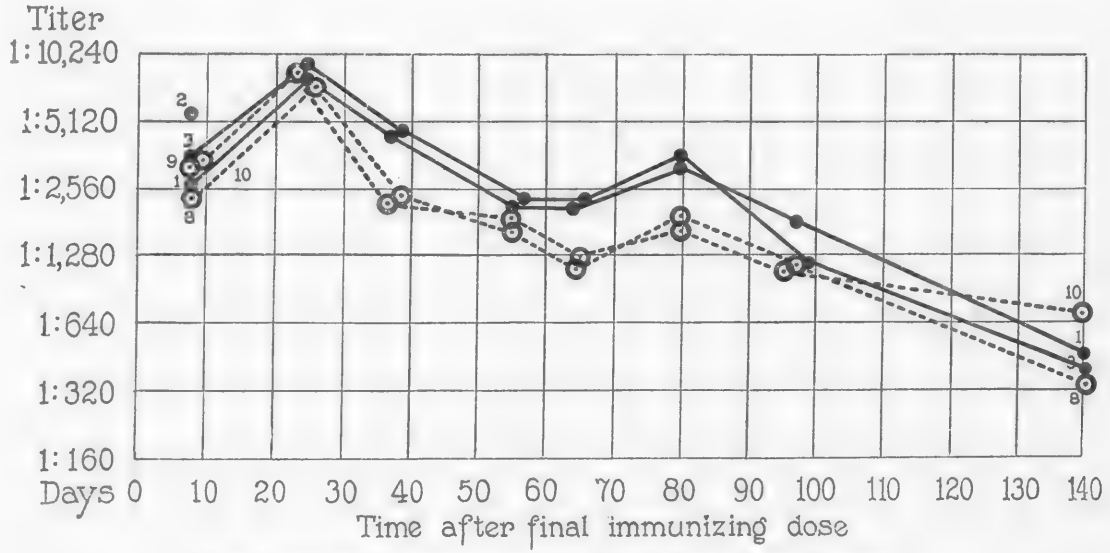
selected and immunization was carried out as described above. Beginning on the day of the first inoculation all animals were fed two medicine droppers full of milk. To the milk of three animals 0.13 gm. of the Burroughs Wellcome whole pituitary gland extract was added. The feeding of milk and of pituitary extract in the same amount of milk was carried out daily until the experiment was finished. This was the method of feeding gland extract adopted by Goetsch (9)



TEXT-FIG. 4. Series 2. Hemolysin titers before and after hypophysectomy.

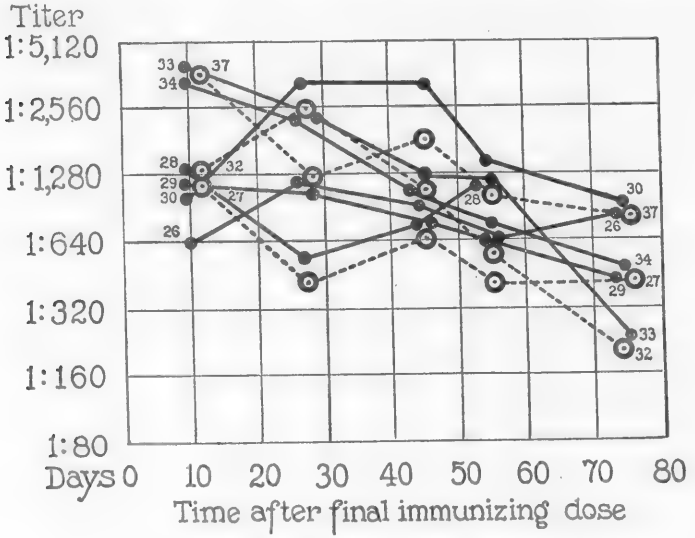
and proved very satisfactory, the animals taking their ration with evident relish. The control animals were fed milk to obviate any criticism of extra nourishment vitiating or influencing the experiments. Beginning 9 days after the last inoculation the agglutinin titer was studied, such studies being then repeated at weekly or biweekly intervals. Text-fig. 5 represents an experimental unit of the above description. The parallelism of normal and pituitary-fed animals is striking.

In another group of animals the gland was given by intraperitoneal injection. 1 gr. of Burroughs Wellcome whole gland extract



TEXT-FIG 5. Series 3. The effect of feeding pituitary extract on the typhoid agglutinin titer. In two animals, only one determination was made because they died from cardiac puncture the day of the first titration.

tablets was used dissolved in 1 cc. of normal saline solution. 1 cc. of normal saline solution was injected at corresponding intervals into the controls. Nine animals were used in these tests, three for controls and six for injection. The animals were first immunized to *Bacillus typhosus*. On the 10th day following the last inoculation their agglutinin titer was determined. This was repeated in 18 days. 10 days later the injection of pituitary extract and salt solution was



TEXT-FIG. 6. Series 3. The effect of repeated intraperitoneal injections of pituitary extract on the typhoid agglutinin titer.

begun and repeated every other day for 3 doses, following which the agglutinin titer was again done. Later the pituitary extract injections were repeated and again subsequent agglutinin titrations carried out. Such an experiment is reported in Text-fig. 6. Further studies varying the amount of extract injected and the intervals between injections were carried out, but no appreciable changes were noticed. As seen in Text-fig. 6 the animals injected with pituitary ran a titer approximate to that of the controls.

SUMMARY.

An operative technique was evolved permitting successful partial hypophysectomy in guinea pigs.

Such animals, when immunized to *Bacillus typhosus*, produced specific agglutinins in the same quantity and at the same rate as unoperated and operation controls immunized at the same time and by the same method.

In guinea pigs previously immunized to *Bacillus typhosus* and hen red blood corpuscles partial hypophysectomy had no effect on the continued production and persistence of typhoid agglutinins, hemagglutinins, and hemolysins.

In guinea pigs immunized to *Bacillus typhosus* both the continued ingestion and the intraperitoneal injection of the whole pituitary gland extract (Burroughs Wellcome) had no effect on the subsepuent agglutinin titers as compared to that of normal animals.

The experiments would appear to show either that the hypophysis does not play an important direct or indirect part in the production of and persistence in the blood of typhoid agglutinins, hemagglutinins, and hemolysins, or that the amount of hypophysis left behind in the operation in order to maintain life is adequate also to exercise the degree of functional influence on these processes which the entire hypophysis conceivably exercises.

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STUDIES UPON EXPERIMENTAL MEASLES.

I. The Effects of the Virus of Measles upon the Guinea Pig.

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Investigations dealing with the nature of the microorganismal excitants of the exanthematous diseases have been numerous during the past two decades, and the present point of view relates the latter to the so called filterable viruses. It is obvious that more precise and definite knowledge of these agents is greatly to be desired.

The nature of the virus of measles has received little attention from the standpoint of research since the transmission experiments of Hektoen[1] showed the existence of the virus in the circulating blood, and those of Goldberger and Anderson[2] proved that the virus is a filter passer. The recent work of Blake and Trask[3] has both extended and confirmed previous knowledge and has appeared at a strategic time when the studies of Sellards[4] had shaken confidence in the earlier experimental findings.

In the investigation of the virus of measles or of the exanthemata as a whole, it is a striking fact that the ape, chimpanzee, and the monkey especially, have been the experimental animals of choice, and they have been chosen because of the greater likelihood of reproducing in them a clinical picture analogous to that of the human disease.

[1] Hektoen, L., Experimental measles, *J. Infect. Dis.*, 1905, ii, 238.

[2] Goldberger, J., and Anderson, J. F., The nature of the virus of measles, *J. Am. Med. Assn.*, 1911, lvii, 971.

[3] Blake, F. G., and Trask, J. D., Jr., Studies on measles. I. Susceptibility of monkeys to the virus of measles, *J. Exp. Med.*, 1921, xxxiii, 385.

[4] Sellards, A. W., and Wentworth, J. A., Insusceptibility of monkeys to inoculation with blood from measles patients, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 57. Sellards, A. W., Insusceptibility of man to inoculation with blood from measles patients, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 257; The reaction of monkeys to the inoculation of measles blood, 311.

Since the work of Inada and Ido[5] on hemorrhagic jaundice, and that of Noguchi on yellow fever, it is evident that the lower type of laboratory animal, namely, the guinea pig, is to be regarded an important host for even highly specialized parasitic microorganisms. The observations of Noguchi[6] on the effects of the virus of yellow fever in the guinea pig have shown that a detailed study of the temperature, the leucocytes, and the clinical phenomena, comprises in this animal an important basis for establishing the existence of infection. This class of observations may be regarded as having contributed to the discovery of *Leptospira icteroides*. While there is no intention of intimating that a leptospira may be the incitant of measles, it appeared to us that if such phenomena could be induced in the guinea pig by the inoculation of measles virus they would provide a far more practical method of handling the experimental measles problem. The use of guinea pigs in experiments on measles has heretofore been limited, and the results have proved unsatisfactory for the reason that the obvious clinical effects looked for were not detected.

As Hektoen and later other investigators demonstrated the presence of the active agent of measles in the circulating blood during the height of the disease, we chose this medium as the virus material with which to attempt transmission to and propagation in the guinea pig. An abundance of material was available, as, during the months of December, 1920, and January and February, 1921, an epidemic of measles prevailed in New Orleans, providing many suitable cases for observation.

EXPERIMENTAL.

The object of the experiments was the determination of the possibility of transmission of measles virus from man to the guinea pig. If transmission could be successfully accomplished, propagation of the virus by passage from guinea pig to guinea pig and a study of the

[5] Inada and Ido, A report on the discovery of the causal organism (a new species of spirochæta) of Weil's disease, *Tokyo Ijishinshi*, 1915, No. 1908. Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., The etiology, mode of infection, and specific therapy of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiii, 377.

[6] Noguchi, H., Etiology of yellow fever. II. Transmission experiments on yellow fever, *J. Exp. Med.*, 1919, xxix, 565.

effect of such passage on virulence, together with a study of the relative infectivity of the passage virus, were next to be attempted. Finally, the plan included the determination of the relative infectivity of blood obtained in various stages of the human disease; namely, prodromal, preeruptive, eruptive, and convalescent.

The blood taken for clinical study was collected under aseptic precautions by means of all glass syringes, immediately placed in sterile flasks containing glass beads, and defibrinated by a rotary motion. Within 15 to 30 minutes after the blood was removed and defibrinated a part was injected into the circulation of the guinea pig. The animal inoculations were made by intracardiac injection of 1 to 2 cc. of material. The guinea pigs were anesthetized with ether and cardiopuncture was accomplished with a No. 24 gauge needle. In order to be certain that the inoculated blood was deposited directly into the heart chambers, removal of a small portion of blood was performed by syringe suction before the introduction of the infecting material.

The method of study after inoculation consisted in twice daily taking of rectal temperatures, daily counting of the total leucocytes, and close inspection of the animals for signs and symptoms of illness during a period of 5 weeks observation. Animals sacrificed at the height of the reaction and those dying subsequent to inoculation were autopsied and a gross and microscopic study of the tissues carried out. Cultures were made from heart's blood and internal organs to determine the presence of intercurrent infection.

The leucocytic count of normal guinea pigs has been made the subject of fairly comprehensive study. Thus, Bethe[7] gives the average number as 7,240 per c. mm., Kurloff[8] as 12,600 per c. mm., and more recently Burnett[9] as 10,897. Hussey[10] is in entire disaccord with

[7] Bethe, M., Beiträge zur Kentniss der Zahl- und Massverhältnisse der rothen Blutkörperchen, *Morphol. Arb.*, 1892, i, 207.

[8] Kurloff, cited by Ehrlich, P., and Lazarus, A., Die Anæmie, in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1901, viii, 56.

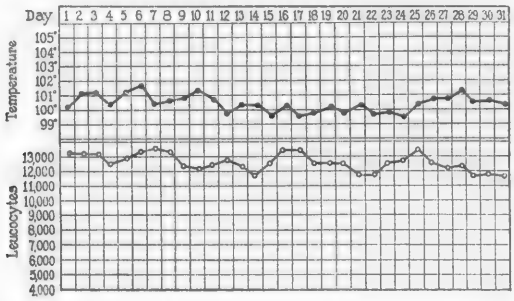
[9] Burnett, S. H., A study of the blood of normal guinea pigs, *J. Med. Research*, 1904, xi, 537.

[10] Hussey, R. G., General leucocytic response of the guinea pig during the reaction of artificial immunity in experimental tuberculous infection, *J. Exp. Med.*, 1921, xxxiii, 337.

these investigators in that he gives 5,247 as the average count on an unstated number of animals.

We have not been able to find in the literature any extensive reference to the normal temperature limits of guinea pigs. We accordingly decided to construct curves of the rectal temperatures of these animals, in order to furnish a basis for the determination of pyrexia.

Experiment 1 (*Preliminary*).—Thirty guinea pigs were used in the experiment, temperatures being determined daily for 31 days. For sake of completeness, leucocytic determinations were made at the same time. Text-fig. 1 represents the composite curves constructed as a result of the experiment.

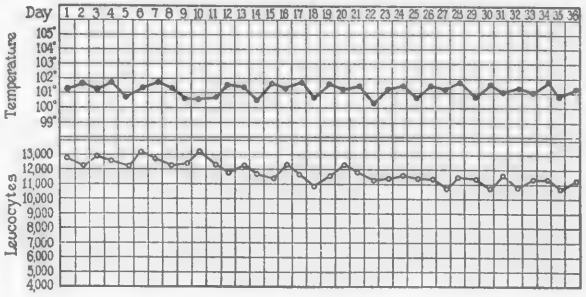


TEXT-FIG. 1. Composite temperature and leucocytic charts of thirty normal guinea pigs.

The results show that the average temperature of the animals in our series, was 100.7°F., the limits being 100° and 101.4°F. In the main experiments, we disregarded as an elevation of temperature any reading below 102°F. The leucocytes for our series averaged 12,250, the limits being 10,750 and 13,750.

Experiment 2 (*Preliminary*).—Since defibrinated blood from cases of human measles was to be injected into the circulation of guinea pigs, it appeared desirable to establish whether or not any reactionary objective signs, temperature increase, or leucocytic changes could be induced by the injection of normal defibrinated blood. Accordingly intracardiac injections were performed on a series of fifteen guinea pigs, defibrinated blood from adults and children being given in quantities varying from 0.5 to 5 cc. Temperature and leucocytic curves constructed in Text-fig. 2 show the effects of these inoculations.

It thus appears that normal defibrinated blood when injected into the circulation of guinea pigs occasions no pyrexial reaction or leucocytic changes. Furthermore no objective signs of illness were to be noted in any of these animals. A number of guinea pigs were sacrificed at periods ranging from 7 to 20 days after inoculation and a histopathological study was made of the various organs. In no case were lesions discernible, except small localized areas of congestion and atelectasis in the lungs, to which we paid no special attention, for as pointed out by Maitland, Cowan, and Detweiler,[11] such



TEXT-FIG. 2. Effects of intracardiac inoculation of normal human defibrinated blood upon temperature and leucocytes of guinea pigs.

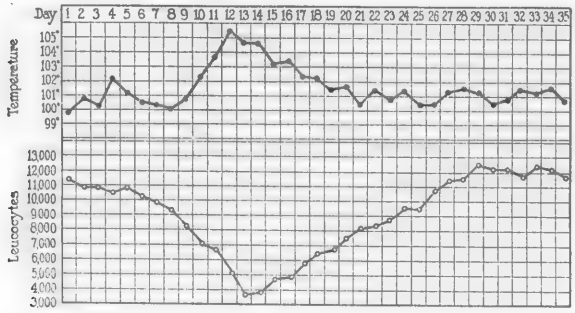
changes are agonal and frequently occur following an anesthetic and death in the guinea pig.

Experiment 3.—The purpose was to determine whether the virus of measles could be directly transmitted from man and propagated in the guinea pig. Blood from seven different cases of measles in the eruptive stage of the disease was employed as the inoculating material. Six of the cases were children ranging in age from 4 to 10 years, and one was an adult. For each experiment of the series, six guinea pigs were used, four for inoculation and two for control. The test animals all received 2 cc. of defibrinated blood containing virus directly into the cardiac circulation, while the controls were given a like quantity of normal blood. In each instance the blood was cultured on ordinary media at the time of injection to exclude bacterial contamination.

[11] Maitland, H. B., Cowan, M. L., and Detweiler, H. K., *Brit. J. Exp. Path.*, 1921, ii, 8.

Of the twenty-eight guinea pigs injected with virus, twenty subsequently showed unmistakable reactions, alike in all respects, which could only be interpreted as indicative of transmission of the infecting material. The control animals remained normal throughout the entire period of the experiment. In general, the effect of the blood containing virus upon the guinea pigs was characterized by a gradual rise of from 2–4° in the temperature, beginning on the 11th to 14th days after inoculation, the animals in the interim remaining well to all outward appearances.

The average number of guinea pigs in each of the seven series that gave a definite reaction was three, or 75 per cent. Perhaps a greater percentage of the animals of this series reacted, but we considered as reactions only those which showed a rise in temperature above 102°C. Coincident with the temperature



TEXT-FIG. 3. Temperature and leucocytic curves after inoculation of human measles blood secured at height of eruption.

elevation the leucocytes dropped until a decided leucopenia, as low as 4,000 cells per c.mm. in a number of instances resulted. The fall in the circulating leucocytes was a more constant sign of the reaction than the temperature rise. While in the majority of the reacting animals the temperature rise occurred coincidently with the leucocytic drop, in six animals there occurred a well marked leucopenia by the 12th day with no coincident temperature rise. It is noteworthy that in the majority of the animals reacting there occurred an initial rise in the temperature on the 2nd or 3rd day after inoculation, unaccompanied by any fall in the leucocytes. This pyrexia was maintained only for a period of 24 hours and usually represented an elevation of 1°F. At first we attributed this initial rise to the foreign protein inoculated; however, animals receiving normal blood showed no such rise, and we are unable at present to offer any explanation. Text-fig. 3 graphically represents the typical temperature and leucocytic changes induced.

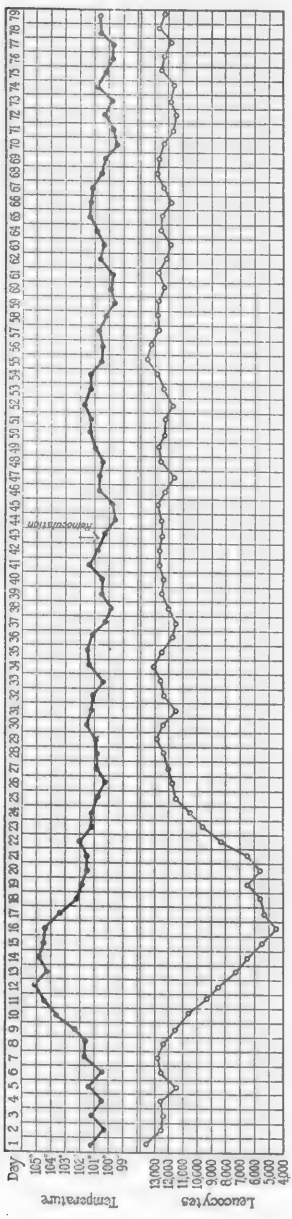
In order to test the effects of reinoculation upon previously reacting animals six recovered guinea pigs were reinoculated with 2 cc. portions of blood secured from human cases of measles at the height of eruption; in none were further pyrexial or leucocytic changes noted (Text-fig. 4).

A number of these animals were sacrificed when the reaction was at its height, and at autopsy the kidney invariably presented microscopic and often gross evidence of acute hemorrhagic nephritis. Blood was removed from certain animals of the series without killing them, and transferred to new guinea pigs for the purpose of virus propagation. Cultural control of the withdrawn blood, which was always carried out, proved negative in all cases for ordinary microorganisms.

Experiment 4.—The purpose was to continue the propagation of the virus by passage from guinea pig to guinea pig. Blood of animals reacting to the direct transfer of measles virus from man was removed by cardiopuncture at the height of the reaction, defibrinated, and injected in 1 cc. quantities directly into the heart chamber of new animals. Seven different passage strains were used and four to six guinea pigs employed. With two strains the transfers were discontinued after the third generation, while with four others the passage was carried to the eighth generation. In these animals the proportion of reactions was greater than with those of Experiment 3. In some series all the guinea pigs reacted in a characteristic manner, in others only part. The reaction was identical with that observed in the direct transmission experiments. After an incubation period of 9 to 10 days there occurred a rather abrupt rise in temperature of 2–4°F. and a coincident fall in the leucocytes. The temperature remained at the peak for 2 to 3 days, gradually subsiding, and reaching normal on the 14th to 16th days after inoculation.

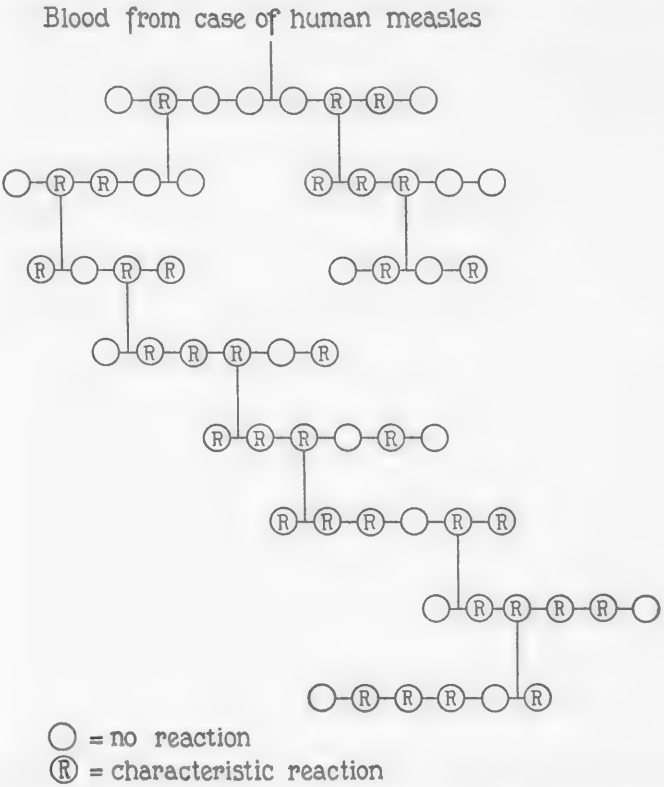
In this experiment it is noteworthy that a number of guinea pigs succumbed presumably as a result of the inoculated virus. Death occurred not infrequently in virus animals of the third generation. Such animals were visibly sick during the period when the reaction was at its maximum; namely, on the 9th to 14th days. They became listless, the hair ruffled, and they remained in the corner of the cage refusing food. Autopsies showed an acute hemorrhagic nephritis which was confirmed by microscopic study. Cultures from the heart's blood, kidney, etc., were always negative. Aside from acute congestion and slight parenchymatous changes, other organs showed nothing worthy of note. The lungs in no instance revealed lesions of any significance. Text-fig. 5 expresses graphically the propagation experiments with a typically reacting passage strain.

Experiment 5.—Since we found that the guinea pig reacted specifically to the intracardiac injections of measles virus, three series of experiments were carried out with a view of ascertaining the relative period of infectivity of the human blood containing virus for the guinea pig. One series of tests comprised twenty-four guinea pigs which were inoculated by the cardiac route with 1 cc. of fresh defibrinated blood that had been secured 1 hour previously from four children exposed to measles by living in close contact with members of their family suffering from



TEXT-FIG. 4. Effects of reinoculation with human defibrinated measles blood secured at height of eruption on temperature and leucocytes of guinea pig which had reacted previously.

the disease. All these children showed 0.5°–1° rise in temperature and some malaise, and two showed slight coryza. Clinically they were classed in the prodromal stage; later all these children developed measles. Subsequently it turned out that the blood was secured 60, 45, 40, and 26 hours, respectively, before the appearance of the eruption. A second series of twenty-four guinea



TEXT-FIG. 5. Showing the continued reaction in guinea pigs through eight successive generations for a single strain of measles virus.

pigs was injected intracardially with 1 cc. of defibrinated blood from four cases of measles in the preeruptive stage. These cases at the time of collecting the blood showed temperatures ranging between 99° and 100°F. and marked coryza, and two showed Kopliks spots. In this experiment the blood injected was withdrawn from the patients 36, 30, 16, and 12 hours previous to the appearance of

the rash. The third series comprised eighteen guinea pigs which received 1 cc. of fresh defibrinated measles blood secured at periods from 24 to 48 hours after the full abatement of clinical symptoms. It is noteworthy that one of the bloods used was obtained from a case previously tested during the eruptive stage, and from which animal inoculations gave typical leucocytic and temperature reactions.

Animals of Series 1, which were to test the infectivity of the blood in the prodromal stage of measles, did not show at any time an elevation in temperature or decrease in the leucocytes though observed daily over a period of 5 weeks. The negative result in all these animals would indicate that the blood of cases of human measles is not infectious for the guinea pig during the so called prodromal period. As further indication that such is the case, some of these animals were subsequently inoculated with 1 cc. of blood containing virus secured during the eruptive stage, with resulting typical reactions. It is possible, though not probable, that the failure of the animals to respond was due to the small amount of the specific excitant which existed in the quantity of blood injected. We are inclined to believe, however, that the virus had not yet invaded the blood at this stage.

With the second series of animals, the purpose of which was to determine the infectivity for the preeruptive stage, very few reacted, and in none were the reactions very definite. In three out of a total of twenty-four there occurred a slight rise in temperature after an incubation period of apparently 2 weeks, but at no time was there an appreciable decrease in the leucocytes. Two of these animals, however, subsequently failed to react when injected with 2 cc. of blood secured from a human case at the height of eruption. This might be considered as acquired protection in the guinea pig produced by a previous infection. At any rate we conclude from this experiment that the blood of preeruptive cases of measles is not infectious for the guinea pig beyond 12 hours preceding the eruption.

In the third series, in which it was attempted to determine how long the blood of the convalescing human case remains infectious for the guinea pig, none of the eighteen animals inoculated with blood collected 24 hours after the temperature had reached normal, even though the rash was still discernible on the patient, reacted in a suggestive manner.

It would then seem that the infectious phase of the human, virus-containing blood for the guinea pig has a range of approximately 54 hours. This time includes in man the latter part of the preeruptive, and the early part of the temperature declining periods, in addition to the period of temperature height and full eruption.

Experiment 6 (Cultural).—We desire at this time to record very briefly the cultural experiments which were performed during the early part of this study. Blood from cases of human measles and from guinea pigs which had previously reacted to inoculation was defibrinated and cultured on a variety of media. For instance, aerobic and anaerobic cultures were made with red agar, chocolate agar, potato blood agar, and Noguchi media both solid and fluid, as substrate. Quantities of blood varying from 0.1 to 1 cc. were used as the inoculating material,

with incubation at various temperatures for periods ranging from 1 to 25 days. No macroscopic or microscopic growths could be discerned. On a number of occasions we encountered peculiar structures resembling somewhat in appearance the globoid bodies of Flexner and Noguchi.[12] We mention this purely for the sake of completeness, as for the present we are entirely disregarding them and are inclined to look upon them as colloidal aggregates.

SUMMARY.

In general it may be said that three striking alterations occurred constantly in the animals reacting to intracardiac injections of blood from cases of measles; namely, pyrexia, leucopenia, and nephritis. The elevation in temperature usually began about the 9th day following inoculation, the rise being fairly abrupt from the normal to 104°F. and above, at which height it remained with slight fluctuations for 3 or 4 days, finally subsiding by lysis. During the period of fever, particularly in the animals of first transmission, often no objective signs of illness were to be noted, the guinea pigs remaining lively and eating as usual. When the animals showed any outward signs these were manifested by a loss of appetite, lusterless and ruffled hair, and indisposition to move even when disturbed. The typical exanthematous signs of human measles were not observed in any of the animals. In some there occurred a slight coryza and watering at the eyes; however, no special account was attached to these signs as they were rather indefinite and inconstant, especially for animals of first transmission.

Coincident with the rise of temperature there always appeared a fall in the total leucocytic count; in some animals the drop in white cells preceded the rise in temperature by 2 to 3 days. The leucopenia, though well defined, varied for animals of the same series. In some the count was as low as 3,600 cells per c. mm.; in no case did we fail to note at least a moderate fall in the leucocytes. The average time for the appearance of this cellular change was 9 to 10 days following inoculation; in other words, the leucocytic reaction seemed to indicate the end of the incubation period. So constant and striking was the leucopenia that we stress this as the most significant feature of the reaction of the guinea pig to the injection of measles blood, particularly since normal blood produced not the slightest change in these

[12] Flexner, S., and Noguchi, H., Experiments on the cultivation of the microörganism causing epidemic poliomyelitis, *J. Exp. Med.*, 1913, xviii, 461.

white elements. The greatest leucocytic depression was observed around the 12th to 14th days, which corresponded approximately to the stage of temperature peak. Following the leucopenia, the leucocytic rise was very gradual, taking on the average 8 days to attain the normal level.

The animals killed at the height of the reaction, or those dying presumably from the effects of the blood containing virus, showed as the most constant lesion gross evidences of acute nephritis. The kidneys in these cases were swollen, cloudy, and congested. In certain guinea pigs there were in addition well defined petechiæ and larger blood extravasations scattered throughout the cortical substance. In the animals dying after inoculation, the kidney alterations were found so constant and characteristic that we regarded them as a special index of the experimental infection. In these animals the hemorrhagic areas were not infrequently 2 to 5 mm. in extent, and when occurring on the surface of the organ produced a separation of the capsule. Occasionally free blood was noted in the pelvis, and in the absence of hemorrhage within the pelvic lining, we assumed that it came from the uriniferous orifices of the calices. Microscopically the kidney sections revealed evidence of acute hemorrhagic nephritis, the hemorrhages for the most part being related to the capillaries of the tufts and the tubules of the pyramids. In the most pronounced cases practically all glomeruli were affected. Every stage from marked dilatation of the capillary whirl to well defined intercapillary blood extravasations that often filled the capsular space, and in consequence partially or completely obliterated the glomeruli, were found. Whether a destruction of the endothelium occurred, could not be determined. The absence of a neutrophilic reaction was of special significance, these elements of acute inflammation being nowhere found associated with the kidney lesion. The blood in relation to the tubules commonly occurred in the lumen of the collecting tubules and in the form of red blood cell casts.

The spleen usually was found enlarged, particularly in the guinea pigs presumably dying as a result of the virus injections. Aside from the usual parenchymatous changes common to toxemias, the other internal organs showed nothing of special note.

CONCLUSIONS.

Guinea pigs react specifically to intracardiac injections of defibrinated blood from cases of human measles.

There is a definite and constant rise in the temperature and a coincident decrease in the total number of leucocytes after an incubation period of 9 to 12 days.

The guinea pig reaction is produced with the human blood only during a certain phase of the disease, which period corresponds approximately to the eruptive stage. 36 hours prior to the eruption and 24 hours after the temperature is normal, the human blood gives rise to no reaction in this animal. The reaction follows with greater frequency in animals inoculated with measles blood obtained at the height of the eruption.

Guinea pigs which react and recover are not susceptible to reinoculation with measles blood if tested over periods of 2 weeks to 3 months after recovery.

Guinea pigs receiving normal human blood injected intracardially do not react with leucocytic or temperature changes.

The constancy of the reaction described leads us to conclude that propagation of the virus is obtained by passage of the blood from infected guinea pig to normal guinea pig; that such passage seemingly increases virulence, since a number of animals thus inoculated die during or subsequently to the peak of the reaction. Apparently death in these cases results from acute hemorrhagic nephritis. Ordinary intercurrent or secondary infection plays no part in these effects as shown by careful bacteriological examination.

Postmortem examinations of animals sacrificed at the height of reaction, and of animals dying, presumably, as the result of the specific infection, show an intense hemorrhagic nephritis, which is a constant pathological finding.

Attempts to cultivate the virus of measles so far have been unsuccessful.

A typical exanthem is not noted in the guinea pig following the intracardiac method of inoculation of human blood containing virus. Coryza and injection of buccal mucous membrane occur in some animals, but are so inconstant as not to be included by us as part of the specific reaction.

While the symptoms and lesions produced in the guinea pig are not entirely analogous to those in man, the constancy of a characteristic reaction after a definite incubation period, together with the successful reproduction of this reaction in subsequent guinea pig to guinea pig inoculations, indicates that the virus of measles is capable of being propagated in the guinea pig, and is pathogenic for this animal.

HEMOLYSIS OF ERYTHROCYTES IN CONTACT WITH GLASS.

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(Received for publication, September 24, 1921.)

In the course of experiments in which it became necessary to make counts of the numbers of red blood corpuscles in various solutions without the use of fixatives it was observed that the cells began to hemolyze as soon as they settled out in the hemocytometer, even though the same cells might remain intact for hours when not in contact with the glass. Hemolysis occurred in some cases so rapidly that it was impossible to make accurate counts. Hemolysis of this sort must have been frequently observed by others, but the literature seems to contain no mention of the fact, nor does any study seem to have been made of the conditions underlying the phenomenon. An investigation of the subject was undertaken with the idea that it might throw some light upon other well known reactions between blood cells and solid bodies, such as the phagocytosis of solid particles, the spreading of leucocytes against solid surfaces, and the disintegration of blood platelets upon contact with foreign bodies.

EXPERIMENTAL.

Contact Hemolysis.

The phenomenon of contact hemolysis may be easily demonstrated in the following manner. A drop of blood is taken from the finger into 25 cc. of 0.9 per cent sodium chloride. The cells are centrifuged out and resuspended in 25 cc. of 0.9 per cent sodium chloride. If a drop of this suspension is mounted on a slide which is not scrupulously clean, the more or less rapid hemolysis of the cells may be readily observed, especially if no precaution has been taken to neutralize the sodium chloride solution which, ordinarily, is as acid as pH 5, even when prepared from chemically pure material. Under

favorable circumstances all the cells may have disappeared in 5 minutes, although there has been no change in the original material. Quantitative data are obtained in the same manner by making frequent counts of the numbers of corpuscles still intact on the slide and in suspension.

TABLE I.

Time Required for 50 Per Cent Hemolysis of Washed Erythrocytes Resting on Glass and in Suspension.

Erythrocytes.	pH	Time required for 50 per cent hemolysis.		
		In suspension.	On glass.	
		hrs.	*hrs.*	*per cent**
Human.	3.5	0.18	0.07	39
	4.0	0.27	0.17	63
	4.0	0.2–0.4	0.10	30±
	5.2	1–2.+	0.60	50–
	6.0	3.+	2.+	—
	9.3	2.90	1.10	38
	10.0	2.+	0.87	50–
Rabbit.	3.3	0.20	0.07	35
	3.5	0.32	0.07	22
	3.6	0.36	0.10	28
	3.8	0.52	0.05	10
	4.0	0.31	0.06	19
	4.2	0.6–1.0+	0.15	25–
	9.6	0.43	0.22	51
	9.7	0.32	0.22	69
	9.8	0.37	0.23	62
	9.8	0.30	0.24	80
	10.05	0.40	0.44	110
Average.....				47

* Time on glass in per cent of time in suspension.

In some cases the hemolysis under the cover-slip was quite irregular in different parts. This is indicated in the tables either by two figures (as 0.26–6.0), showing the approximate extent of the irregularity, or by a minus sign, indicating that the counts were made where hemolysis was slowest.

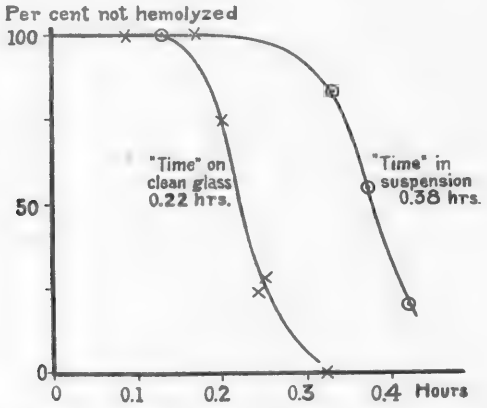
The results of eighteen such experiments with washed rabbit and human erythrocytes are given in Table I. In using human blood, approximately 0.01 cc. was taken from a finger-tip in a calibrated capillary pipette, washed once in 25 cc. of 0.9 per cent sodium chloride, and resuspended in a similar volume of isotonic salt solution. Mix-

tures of varying pH were prepared by taking 5 cc. of this suspension (in some experiments only 1 cc.) plus 1 to 5 cc. of 0.004 M sodium hydroxide or 0.005 M hydrochloric acid in 0.9 per cent sodium chloride plus enough 0.9 per cent sodium chloride to make 15 cc. The pH was measured by a Bovie hydrogen ion potentiometer and a Clark hydrogen electrode. In experiments on rabbits, 0.3 cc. of blood was taken from the ear vein of a rabbit into a pipette previously rinsed in a 0.5 per cent solution of sodium oxalate in 0.9 per cent sodium chloride to delay clotting, centrifuged in 50 cc. of 0.9 per cent sodium chloride, and resuspended in a similar volume of salt solution. Mixtures were then prepared as for human blood, with the exception that usually only 1 cc. of cells was used.

To determine the rate of hemolysis on glass, a cover-slip was supported on the slide at four points by fragments of another cover-slip; and the mixture was allowed to run under the cover-glass, which was then sealed with paraffin. Counts were made at once over equal areas as measured by an ocular micrometer disc ruled in squares. Frequent counts were made during the experiment, the points on the slide counted being chosen more or less at random in order to average to some extent the variations sometimes observed even under the same cover-slip. The course of hemolysis in the remainder of the mixture, left in suspension in the test-tube, was followed simultaneously. It was thought advisable, for the sake of uniformity, to do this by counting the number of intact corpuscles in a given volume on a hemocytometer. Ordinarily about 200 cells were counted for each determination. The results were then calculated in per cent of the original, plotted on coordinate paper, and the time necessary for 50 per cent hemolysis was determined graphically. Text-fig 1. shows two typical curves having the characteristic S shape of hemolysis curves. The time is determined most accurately at the 50 per cent mark, the point of most rapid hemolysis.

For "clean glass" experiments the slides were first thoroughly washed by the laboratory assistant by the usual methods, including treatment with soap and water, cleaning solution, and finally drying with a clean towel. For uniformity they were again allowed to remain in cleaning solution for a time, were rinsed in hot running tap water for 10 to 20 minutes, then in boiling distilled water, and were

finally stacked on a glass rack where they dried in less than a minute. During the final stages of the process they were handled only with glass tweezers. This method has the advantage of avoiding the grease which inevitably collects on the cleanest towel and it saves the labor of drying. It is easy to demonstrate the presence of what is probably a film of grease on a slide by scraping the surface gently with a pair of tweezers held perpendicularly. On a perfectly clean slide a distinct squeak is heard. On slides cleaned by the usual



TEXT-FIG. 1. Curves showing the percentages of washed rabbit erythrocytes still intact at various times after mixing. The time necessary for 50 per cent hemolysis of these cells in suspension (pH 9.8) was 0.38 hours, while the hemolysis time for the same cells resting on glass was only 0.22 hours.

methods involving wiping, there is usually sufficient grease to act as a lubricant so that the tweezers move smoothly over the surface.

The figures in Table I show that hemolysis of washed human and rabbit erythrocytes which are resting on clean glass occurs in less than one-half the time necessary for the cells in suspension. That this cannot be due to small amounts of alkali is shown by the fact that the phenomenon occurs at the point of acid hemolysis as well as at the point of alkali hemolysis. It is, if anything, more pronounced in acid solutions.

Since it was most natural to connect this phenomenon with the clotting of blood on contact with foreign bodies and the disintegration of blood platelets, it seemed advisable to see whether the disintegration of the erythrocytes could be prevented by a thin film of vaseline, paraffin, or paraffin oil on the slide. In Table II the hemolysis time of washed human erythrocytes in suspension is compared with the hemolysis time on a slide covered with a thin film of vaseline. The vaseline was taken from a solution in ether used in the laboratory for coating cannulas to prevent clotting. As on clean glass, the erythrocytes hemolyze more than twice as rapidly on vaseline as they do in suspension. Many of the experiments on vaseline were done simultaneously with experiments on clean glass, with the same mixtures. In such instances, when a direct comparison between vaseline and glass was possible, no significant difference between the two was found. The film of vaseline was just visible to the naked eye; and the microscope revealed many small drops of vaseline, which rounded up in contact with the water and sometimes became hardly distinguishable from blood corpuscles.

TABLE II.

Time Required for 50 Per Cent Hemolysis of Human Erythrocytes on a Vaselined Slide and in Suspension.

pH	Time required for 50 per cent hemolysis.		
	In suspension.	On vaseline.	
	hrs.	*hrs.*	*per cent*
3.3	0.20	0.07	35
3.5	0.18	0.10	55
4.0	0.27	0.17	63
6.0	3.+	2.+	—
10.0	2.+	0.60	30−
10.3	1.+	0.37	37−

Table III gives the results of another experiment with washed rabbit corpuscles in which the time of hemolysis on paraffin and paraffin oil was compared with that in suspension. The paraffin oil ("highest purity"), like the vaseline, offers no protection against hemolysis. The results with paraffin are more irregular, but here too, in most

cases, hemolysis is more rapid in the sedimented cells than in those suspended. There seems to be some other factor involved in the case of paraffin, however, and to some extent with paraffin oil, because the time curves of hemolysis have frequently an abnormal shape, indicating that hemolysis begins very rapidly and stops short of completion, leaving part of the cells intact, sometimes for hours, after all the suspended cells have disappeared. The reason for this abnormality is not apparent. The paraffin or paraffin oil was applied to the slide with a clean cloth, thus producing a thin film barely vis-

TABLE III.

Time Required for 50 Per Cent Hemolysis of Rabbit Erythrocytes on Films of Paraffin and Paraffin Oil, and in Suspension.

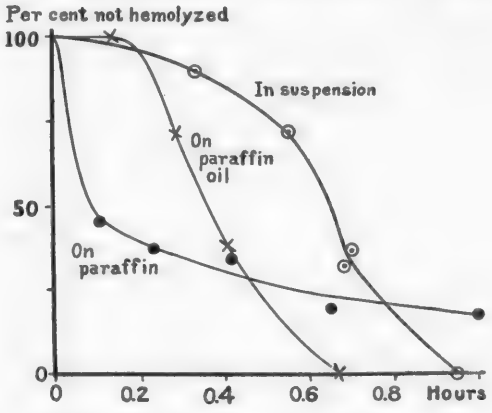
pH	Time required for 50 per cent hemolysis.				
	In suspension.	On paraffin.		On paraffin oil.	
	hrs.	*hrs.*	*per cent*	*hrs.*	*per cent*
3.3	0.20	0.14*	70	0.08*	40
4.0	0.44	0.26–6.0	59+	0.10	23
4.0	0.45	0.12	27	0.14	31
4.2	6.0	6.00	100	0.60	10
9.7	0.56	0.13	23	0.25–0.35	45–53
9.7	0.68	0.09–	13–	0.36–	53–
10.3	0.38	0.38	100	0.18–	47–
11.05	0.15	0.10	67	0.04	27
11.4	0.07	0.03–0.08	42+	0.05	70

* Not completely hemolyzed until after the control, the shapes of the curves being different as explained in the text.

ible to the naked eye but very evident under the microscope—as ridges in the case of paraffin or as drops in the case of paraffin oil. A typical experiment of this series is plotted in Text-fig. 2.

While contact hemolysis does occur with clean glass, it is much more pronounced with slightly soiled glass. Text-fig. 3 represents the results of a series of experiments in which the hemolysis of rabbit erythrocytes on paraffin, paraffin oil, clean glass, and in suspension is compared with the time necessary for hemolysis of the same cells in contact with a soiled slide. The slides were first washed clean by

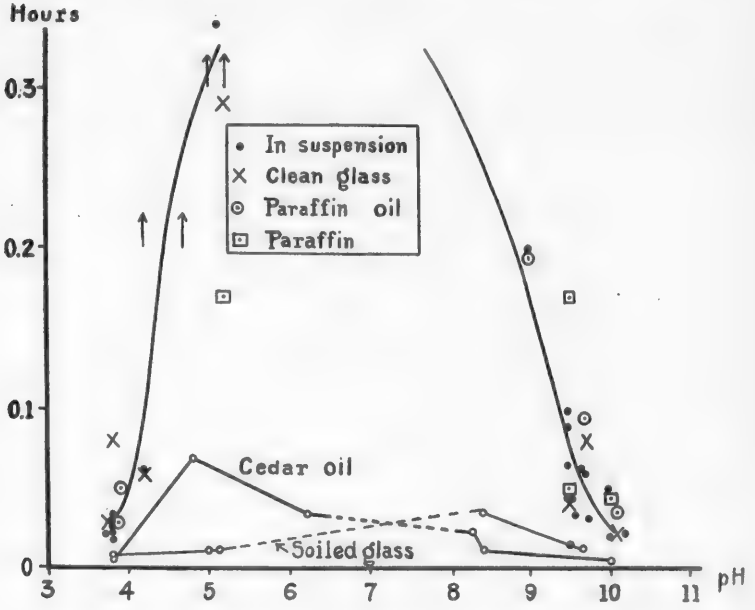
the usual method and were then wiped with a cloth which had been rubbed once on the hair but which was otherwise clean. This treatment produced a dimly visible film on the surface of the glass. The extremely rapid hemolysis is very evident from the figure. A similar experiment, with slides coated with a specimen of cedar oil used for immersion lenses also shows abnormally rapid hemolysis. There was no assurance of the purity of this oil, however, and the result is not of great significance. This figure shows that there is no constant difference between the rate of hemolysis in suspension and the rate of hemolysis on glass, paraffin, or paraffin oil. The apparent dis-



TEXT-FIG. 2. Curves showing the rate of hemolysis of a suspension of washed rabbit cells (2,200 per c.mm.) at pH 9.7 resting on films of paraffin and paraffin oil, and in suspension.

crepancy between this experiment and those previously cited is due to the serum content of the mixture, since the cells were not washed before use. Allowing 40 per cent for the volume of the corpuscles in whole blood, it may be calculated that the serum content of the unwashed cells in the final mixture (5 cc. of cells to 10 cc. of hydrochloric acid or sodium hydroxide) was 0.12 per cent of the normal serum content of blood. Even with this small concentration of serum, contact hemolysis is prevented to a considerable extent on clean glass, though in some experiments it was still evident, particularly in acid solutions. The results of all available comparisons on

clean glass are given in Table IV. In not a few instances the cells on the glass hemolyze even less rapidly than those in suspension. Comparison with Table I reveals the striking effect of small amounts of serum in hindering contact hemolysis.



TEXT-FIG. 3. Curves showing the time necessary for 50 per cent hemolysis of rabbit erythrocytes in 0.12 per cent serum in solutions of various pH values. The rate of hemolysis in suspension is compared with the rate of hemolysis when the cells were resting on clean glass or glass covered with films of paraffin, paraffin oil, cedar oil, or dirt obtained by rubbing a clean cloth on the hair. Arrows indicate that the hemolysis time was greater than the value indicated. Figures showing the hemolysis time in suspension and on clean glass under these conditions are given in Table IV.

In some of the experiments with serum, unwashed cells were used; while in others an equivalent amount of serum was added to washed cells. No alteration in behavior due to the washing was detected.

Further experiments would be desirable in order to see whether the effect of serum is due to the calcium content and whether it can

be destroyed by heating. One experiment was tried to make sure that it was not due to the small amount of oxalate present from rinsing the pipette before taking the blood, but no change in the rate of hemolysis was detected from twice the usual amount of oxalate in the same volume of sodium chloride without serum. In one experiment 0.02

TABLE IV.

Time Required for 50 Per Cent Hemolysis of Rabbit Erythrocytes in 0.12 Per Cent Serum on Clean Glass and in Suspension.

pH	Time required for 50 per cent hemolysis.		
	In suspension.	On glass.	
	hrs.	*hrs.*	*per cent*
3.3	0.24	0.08	33
3.3	0.23	0.15	65
3.4	0.23	0.15	65
3.8	0.40	0.27	68
3.8	0.35	0.27	77
3.8	0.22	0.5–1.0	228+
3.8	0.28	0.28	100
3.8	0.25	0.22	88
4.0	0.17	0.26	153
4.0	0.18	0.22	120
4.0	0.22	0.27	122
4.2	2.+	2.+	—
4.2	0.64	0.6	94
4.7	2.+	0.8–1.2	50–
5.2	3.+	2.8	90–
6.4	1.+	1.+	—
9.5	0.44	0.43	98
9.7	0.54	0.80	140
9.7	0.42	0.42	100
9.8	0.36	0.36	100
9.9	0.24	0.23	96
10.0	0.35	0.41	117
10.2	0.20	0.25	125

per cent of serum was found to prevent hemolysis on glass almost completely, which otherwise took place in about half an hour. This was probably largely due to the buffer action of the serum. No measurements of the pH were made in this experiment.

In a few preliminary experiments it was found that 2 per cent dextrose and 0.5 per cent acacia definitely inhibited hemolysis of human

red cells at pH 5.2, while 0.25 per cent acacia failed to do so. More determinations are necessary to establish this as a fact. Interest in these substances arises from the work of Rous and Turner (1), who recommend dextrose-Locke's solution for preserving red cells and gelatin to protect them from injury during centrifuging. Acacia might be expected to resemble gelatin in this respect.

In view of the hemolyzing effect of small amounts of impurities on the glass it is evident that the interpretation of the results is always open to the possible but improbable objection that the glass was not perfectly clean. It was not thought practicable, however, as a routine measure in such a large number of determinations to subject the glass to more extended treatment, such as rubbing with grease-free diatomaceous earth, etc. It is evident, nevertheless, that contact hemolysis is a phenomenon which must occur frequently in experiments on the preservation of red blood corpuscles *in vitro* (1). Also, the hemolysis often observed in centrifuging washed red blood corpuscles is doubtless of a similar nature. In all such experiments it is essential that the pH be controlled more accurately than has usually been considered necessary, since the hemolysis limits are much narrower for cells in contact with glass. The usual acidity of chemically pure preparations of sodium chloride (pH 5.0) and the alkalinity of sodium citrate solutions make this particularly advisable.

A number of experiments were tried with fresh mica surfaces, in the hope that they might be found cleaner than paraffin or glass, but the result was even more irregular. In some areas on the slide the cells hemolyzed much more rapidly, in others somewhat less rapidly than the control cells in suspension. On the whole, it may be said that contact hemolysis occurs on mica as well as on the other surfaces used.

In explaining these results three possibilities are open. In the first place, one thinks of soluble materials coming from the glass. Thus Hausmann and Kerl (2), in studying oligodynamic hemolysis by various metallic plates, observed that hemolysis occurred also around a glass rod left in the plate of agar containing the red blood corpuscles, and attributed the results to alkali given off by the glass. This explanation, however, seems excluded here because of the fact that contact hemolysis occurs in acid as well as in alkaline solutions.

Another proposal is that of Schwyzer (3), who believed that the formation of rolls by red blood corpuscles in contact with glass was due to a neutralization of their electric charge. This explanation is also somewhat improbable in view of the fact that contact hemolysis occurs on both sides of the isoelectric point of the red blood corpuscles (pH 4.7, as given by Coulter (4)).

A more useful working hypothesis seems to be that contact hemolysis is the result of an attempt on the part of the corpuscle or, perhaps, some ingredient of the corpuscles, as the lipoids, to spread on the glass by surface tension, thus accelerating the disintegration of the structure. It may be supposed that this spreading would occur in neutral as well as in acid and alkaline solutions if it were not for the more or less rigid structure of the corpuscle.[1] This conception is readily correlated with the experiments of Bechhold (5), who observed that when red blood corpuscles were shaken with clay or kieselguhr, the particles of the latter could be observed sticking to the outside of the cells. On shaking the mixture vigorously enough to detach these particles, hemolysis occurred. His interpretation was that the particles tore away with them parts of the cell (he suggests lipoid), thus serving to break up the intimate association of lipoid and protein upon which he conceives the rigidity of the corpuscle to depend. The rigidity of the cells, which keeps them disc-shaped, is obviously reduced in alkaline solutions when they round up. During acid hemolysis on the glass the cells are larger in diameter, either because of swelling (Price-Jones (6)) or because they spread out on the glass into a hemispherical shape. Simultaneously they become lighter in color and focus the rays of light at a higher point than do normal cells. This may be due to a more or less hemispherical shape (such as obtains in a cell which spreads out), or to a lower refractive index, or to both.

If this hypothesis were true, the erythrocytes might be expected to stick to the glass most firmly in those solutions in which they show contact hemolysis to the greatest extent. From a few experiments on the stickiness of erythrocytes, which are described later, it may be

[1] This seems somewhat more probable than the alternative assumption that the cells are not pulled out by surface tension at neutrality as strongly as in acid or alkaline solutions.

concluded that the cells are more sticky in acid solutions and less so in alkaline than they are at neutrality. This observation clearly supports the idea that contact hemolysis, in the acid range at least, is due to a spreading out of the cell. If the same conception is to be applied to alkaline solutions in which hemolyzing cells are, if anything, smaller than normal and in which their stickiness is also less, it must be assumed that some ingredient of the cells, as the lipoids, is spreading invisibly. Since there is as yet no experimental basis for this idea, speculation is futile. It may be pointed out, however, that the flotation processes for the separation of different ores depend in general upon the greater tendency of certain particles of ore to stick to oil drops, suggesting by analogy a greater attraction of the glass for lipoids than for other parts of the cell. However that may be, some readjustment of the cellular equilibrium would be expected, from ordinary principles of surface tension, when a complex system of different phases like a red blood corpuscle comes into contact with a solid body. While this readjustment may not have a detectable effect on a normal cell, it may well accelerate disintegration of a cell which is already at the point of acid or alkaline hemolysis.

It is possible that differences in the sensitivity of erythrocytes to contact with glass may occur between species or between individuals, though no attempts have been made to detect them. The method, however, is not very well suited for the measurement of small variations. One is always confronted by the possibility of invisible films of impurities on the glass which destroy the constancy of the results.

Experiments on Stickiness of Erythrocytes.

Experiments on the stickiness of erythrocytes were undertaken by two methods. In the first, the erythrocytes were allowed to settle out in a vessel made by sealing, with paraffin, a short glass cylinder, 2 cm. in diameter and 3 cm. long, to a microscope slide, the other end being closed by a rubber stopper with a capillary air vent. After an hour or two the vessel containing the supernatant solution and the sedimented cells was rotated on a motor ten or fifteen times in as many seconds about a horizontal axis, thus permitting the circulating liquid to wash off a certain proportion of the cells. This proportion was determined by counting the cells washed off and those still sticking on in known volumes and areas respectively.

The effect of pH on the stickiness of washed rat erythrocytes as measured by this method in two experiments is shown by the following figures.

pH	Erythrocytes stuck on slide.	
	A	B
	per cent	*per cent*
5	17	15
7	0	9
9	0	0

The H ion concentration was maintained by buffer solutions of phosphates in $\frac{M}{60}$ solutions. At pH 5, in both cases, many hemolyzed cells, which had not been washed off when the vessel was rotated, could be dimly seen on the slide. This shows that after contact hemolysis in acid solutions the remains of the cells are stuck to the slide and indicates that contact hemolysis in acid solutions may be due to a spreading of the cells.

In another similar experiment, in which there was apparently no hemolysis, no cells were found sticking to the slide at pH 7 or 9, while there were 700 per sq. mm. at pH 5.

Further measurements by the same method with varying concentrations of serum indicate again an apparent relation between contact hemolysis and stickiness. The results were as follows:

Serum.	Erythrocytes.		
	Washed off.	Stuck on slide.	Remainder, hemolyzed.
per cent	*per cent*	*per cent*	*per cent*
0.00	43 ± 9	10	47
0.17	100 ± 6	0	0
0.35	83 ± 7	0	17
0.70	85 ± 7	0	15

In the presence of serum, no cells stick to the glass and there is no appreciable hemolysis (0, 17, and 15 per cent). Without serum, 57 per cent of the cells are either stuck to the slide or hemolyzed, hemolysis being probably the end-result of sticking. The solution

of 0.9 per cent sodium chloride, used to dilute the serum, was carefully neutralized. Hemolysis was doubtless accelerated by contact with the glass, which lasted in this instance for $1\frac{1}{2}$ hours at 25°C.

The other method by which the stickiness of erythrocytes was measured consisted in allowing a suspension of red blood corpuscles in the solution under investigation to run under a cover-slip, which was supported at four points on a microscope slide by small fragments of a cover-slip, as in the experiments on contact hemolysis. The edges of the cover were sealed with paraffin. After a certain interval of time (5 to 15 minutes) the preparation was inverted and the percentage of cells which were stuck to the slide firmly enough to prevent them from falling off was determined by counting the numbers of cells observed over equal areas on cover and slide. In acid solutions almost no cells drop off no matter how long a time is allowed.[2] In alkaline solutions somewhat variable results are obtained, depending upon the time intervals allowed before and after inverting the preparation. This variation is probably due to the fact that it takes some time to reach equilibrium in alkaline solutions. If the suspension is mounted as soon as mixed and inverted as soon as the cells have settled out, it may take 15 minutes or an hour for the cells to drop off. If, however, 5 minutes are allowed between the time of mixing and the time of mounting, or if 15 minutes are allowed between the time of mounting and the time of inverting, a large proportion of the cells usually drops off during the first 5 minutes after inverting. Two series of experiments by this method are summarized in Table V. In Experiment A, the cells were left in suspension 5 minutes after mixing, to reach equilibrium, and the counts were then made within 5 minutes after inverting. In Experiment B, the cells were mounted immediately after mixing and inverted within 5 minutes, but counts were not made for 15 minutes after inverting. To avoid further complications, this study was limited to those cases in which hemolysis had not begun during the time of the observations. The pH

[2] The behavior of erythrocytes in this respect reminds one strikingly of the device used by Perrin in determining Avogadro's constant by observations on a gamboge emulsion. Acidification of the solutions caused the globules of gamboge to stick to the walls of the vessel, where they could be easily counted (Perrin, J., Die Atome, Dresden, 1914).

values were determined electrometrically and were varied by the addition of 0.005 M solutions of sodium hydroxide and hydrochloric acid in 0.9 per cent sodium chloride. The cells were obtained by centrifuging a few drops of blood from a pricked finger in a known volume of salt solution. When the cells were not washed, the concentration of serum present was calculated from the number of erythrocytes present as determined by the hemocytometer, allowing 40 per cent for the volume of the corpuscles in fresh blood. The results obtained by this method as given in Table V confirm those obtained by the previous method, showing that human erythrocytes are more sticky in acid solutions.

TABLE V.

Effect of pH on the Percentage of Erythrocytes Sticking to Glass.

Experiment A.		Experiment B.	
pH	Erythrocytes stuck on slide.	pH	Erythrocytes stuck on slide.
	per cent		*per cent*
4.1	100	5.5	79
5.2	92	6.5	62
5.6	80	8.0	13
8.0	20	8.8	5
10.0	29	9.7	11
10.1	2		

Experiment A.—Cells in 0.05 per cent serum. They were allowed to run under a cover-slip on a microscope slide 5 minutes after adding sodium hydroxide or hydrochloric acid. 5 minutes later the slide was inverted. After another 5 minutes the percentage sticking on the slide was determined by counting.

Experiment B.—Cells in 0.9 per cent sodium chloride, no serum. They were mounted on the slide immediately after mixing, and counts were not made until 15 minutes after inverting. In alkaline solutions many drop off during this longer interval unless they are allowed time to come to equilibrium after mixing and before mounting. Variations less than 10 per cent are not significant.

SUMMARY.

1. Washed erythrocytes, in both acid and alkaline solutions, hemolyze more rapidly when allowed to settle out on a clean microscope slide than when kept in suspension.

2. This is also true on slides coated with paraffin, paraffin oil, or vaseline, and on mica surfaces.

3. The presence of as little as 0.1 per cent serum inhibits such contact hemolysis, particularly in alkaline solutions.

4. Contact hemolysis is most marked on slightly soiled glass, and may occur so rapidly with unfixed cells in a hemocytometer that accurate counts are rendered impossible.

5. Erythrocytes are more sticky than normally in acid solutions and less sticky in alkaline solutions.

6. The increased stickiness of erythrocytes in acid solutions and their larger size during contact hemolysis in acid media provide some experimental evidence for the view that contact hemolysis is to be correlated with an attempt on the part of the corpuscles, or some ingredient of the corpuscle, to spread on the glass after the manner of leucocytes and invertebrate blood cells.

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